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14. ABSTRACT

Mutations have been recently identified in the EphB2 receptor gene in prostate cancer suggesting that EphB2, a member of the large Eph receptor tyrosine kinase family, is a tumor suppressor in prostate cancer. Consistent with a tumor suppressor activity, we found that EphB2 is more highly expressed in non-transformed BPH-1 prostate epithelial cells than in several prostate cancer cell lines. Furthermore, EphB2 expression was rapidly lost in stably transfected DU145 prostate cancer cells, suggesting that EphB2 has detrimental effects on cell growth and/or survival. We have also uncovered a novel tumor suppressor pathway downstream of the related EphA2 receptor in prostate cancer cells. We found that EphA2 inhibits the Akt-mTOR pathway, a pathway well known to play a critical role in prostate cancer pathogenesis, and are investigating the mechanisms underlying this important function of EphA2. The tumor suppressor activities of the Eph receptors in prostate cancer represent an important area of investigation that will help understand the pathogenesis of this disease and guide the design of novel diagnostic and therapeutic strategies.

15. SUBJECT TERMS
Eph receptor, ephrin, tumor suppressor, signal transduction

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INTRODUCTION

Loss of function of tumor suppressor genes and increased function of tumor-promoting genes are critical steps in the development and progression of cancer. It is therefore important to identify these genes and understand how they affect cancer progression in order to develop new treatments. Inactivating mutations in the EphB2 gene that were identified in clinical prostate cancer samples but not in normal tissue have provided intriguing clues suggesting that the EphB2 receptor, a member of the large Eph receptor tyrosine kinase family, is a tumor suppressor in prostate cancer. Consistent with this, forced expression of EphB2 was shown to suppress the growth of cultured prostate cancer cells that lack EphB2 expression (1). Furthermore, a nonsense mutation in the EphB2 gene has been recently associated with prostate cancer risk in African American men with a positive family history (2). Several mechanisms of EphB2 inactivation in colorectal tumors have also been recently reported, supporting the hypothesis that EphB2 functions as a tumor suppressor (3). However, the signaling mechanisms involved are unknown.

Our work with another Eph receptor in breast cancer showed that activation of Eph receptor signaling pathways by their ligands, called ephrins, can widely inhibit the malignant properties of cancer cells (4). Our hypothesis is that signaling pathways activated by EphB2, and other Eph receptors, inhibit the malignant properties of prostate cancer cells. Importantly, since submission of this application several reports have appeared in the literature highlighting the potentially critical role of different Eph receptors in the pathogenesis of prostate cancer (5-7). Prominent among them is the EphA2 receptor. EphA2, although highly expressed in prostate cancer, has been shown to inhibit the Ras-MAP kinase oncogenic pathway when activated by ephrin-A ligands in prostate cancer cells (8-12). The tumor suppressor nature of ephrin-dependent EphA2 signaling in vivo has been further supported by studies using a mouse skin cancer model (11). EphA2 has also been recently identified as a prostate cancer biomarker, whose expression correlates with sensitivity to the cancer drug dasatinib (13). Interestingly, EphA2 and EphB2 share the same chromosomal location at 1p36.1 (14). Therefore, the loss of heterozygosity reported for this locus in prostate cancer likely decreases not only EphB2 expression (1) but also EphA2 expression.

Our work supported by this award has led to the identification of a novel tumor suppressor pathway triggered by Eph receptors upon ephrin stimulation of prostate cancer cells. This tumor suppressor pathway causes the inhibition of the serine/threonine kinase Akt, a kinase whose activity has been shown to be critically important in prostate cancer development and progression (15-19).

BODY

In the course of this project, we confirmed that transfected EphB2 drastically inhibits DU145 prostate cancer cell growth. While supporting the hypothesis that EphB2 has tumor suppressor activity in prostate cancer, this growth inhibition made it difficult to perform some of the proposed experiments with EphB2-transfected DU145 cells. On the other hand, the experiments to compare the effects of EphB2 with those of other Eph receptors expressed in prostate cancer uncovered a novel ephrin-dependent tumor suppressor activity. This activity leads to dramatic inhibition of a pathway with a critical role in prostate cancer: the Akt-mTOR pathway (15-19). Inhibition of Akt-mTOR, together with the previously characterized inhibition of the Ras-MAP kinase pathway, likely plays a major role in the tumor suppressor activities of the Eph receptors in prostate cancer. Ephrin-dependent activation of Eph receptors can overcome Akt activation due to loss of the Pten tumor suppressor, a loss that often drives prostate cancer progression. Therefore, it is critically important to elucidate the mechanisms linking ephrin-dependent Eph receptor activation to inhibition of Akt.

In the last progress report, which was found to be scientifically acceptable, we reported this novel development and expressed the intention to focus on the characterization of the mechanisms by which Eph receptors cause such a dramatic downregulation of Akt activity. During the last year, we have performed many informative experiments with PC3 cells stimulated with ephrin-A1 Fc, which have excluded many possible signaling mechanisms. We have therefore requested, and have been granted, a one year extension of this grant without additional funding, to continue this work and further characterize how EphA2 inhibits Akt as well as to determine whether EphB receptors such as EphB2 also inactivate Akt through a similar mechanism. Following up on a comment of the reviewer of our previous progress report, we are providing a revised Statement of Work to reflect these new developments in the project, which were already outlined in the previous (approved) progress report.

Aim 1. Determine whether the growth-suppressing activity of EphB2 and other Eph receptors in prostate cancer cells depends on activation by ephrin ligands

Task 3. Determine the effects of transiently transfected EphB2 and other ligand-activated Eph receptors on prostate cancer cell growth.

As stated in the previous progress report, we found that transfection of EphB2 severely inhibits the growth of DU145 cells (consistent with the hypothesis that EphB2 is a tumor suppressor gene in prostate cancer). Experiments are in progress to examine whether this is a general effect of EphB2 in prostate cancer cells by transfecting low levels of EphB2 in PC3 cells to increase the levels of this receptor as well as by stimulating endogenously expressed EphB2 and other EphB receptors with ephrin-B ligands. In the last year we have also performed extensive experiments showing that ephrin-A1 Fc stimulation of PC3 cells inhibits their 2D growth on tissue culture plates (Fig. 1) and 3D growth in focus formation assays (Fig. 2) and spheroid assays in Matrigel (Fig. 3). We attribute this tumor suppressor effects to the ephrin-A1-dependent activation of EphA2, which is the main EphA receptor present in the PC3 cells.

To complete this task, experiments in progress involve using the MTT assay and focus formation assay with DU145, LNCaP and PC3 cells to measure the effects of ephrin-B1 Fc and ephrin-B2 Fc stimulation (to activate EphB receptors) and ephrin-A1 stimulation (to activate EphA receptors). The DU145 cells lack EphB2 and the LNCaP cells lack EphA2, and will therefore be useful to determine if ephrin-induced activation of other EphB receptors expressed in the DU145 cells or other EphA receptors expressed in the LNCaP cells can also inhibit growth.

Aim 2. Characterize the effects of EphB2 and EphA2 signaling pathways on prostate cancer cell survival, proliferation, migration and invasion

Task 4. Identify tumor suppressor signaling pathways stimulated by ephrins in prostate cancer cells.

Inhibition of cell growth by forced expression of EphB2 in DU145 cells is consistent with tumor suppressor activities, and tumor suppressor activities in prostate cancer have also been reported in the literature for the EphA2 receptor (10). Therefore, we proceeded to investigate the pathways activated downstream of EphB2 and EphA2 in prostate cancer cells stimulated with ephrin ligands. These experiments have revealed inactivation not only of the Ras-MAP kinase pathway but also of Akt and the downstream mTOR kinase. We have focused our recent efforts on the characterization of the novel EphA2-Akt signaling pathway that we have identified downstream of Eph receptors in prostate cancer cells.

EphA2 activation by ephrin-A1 dramatically inhibits the Akt-mTOR pathway. The activity of the serine/threonine kinases Akt and mTOR is of major importance for cell growth and

transformation (20). Akt activates mTOR and other downstream effectors that play an important role in oncogenesis by promoting cell cycle progression and cell survival and inhibiting the onset of cellular senescence (20-22). Typically, growth factor receptors activate this pathway through PI-3 kinase, which phosphorylates the phospholipid PI(4,5)P2 to produce PI(3,4,5)P3 (Fig. 4). Binding to PI(3,4,5)P3 causes the relocalization of the serine/threonine kinase Akt to the plasma membrane, where Akt is activated by phosphorylation at T308 and S473. Activated Akt in turn phosphorylates and inactivates TSC2 (also known as Tuberin), which is a GTPase-activating protein for the Ras family protein Rheb. This leads to activation of Rheb and its downstream target mTOR (mammalian target of rapamycin). A well characterized target of mTOR is p70 S6 kinase, whose phosphorylation at T389 is a sensitive readout for mTOR activity. The Akt-TOR pathway is often activated in cancer cells, including PC3 and LNCaP, due to the loss of the tumor suppressor Pten, which is a lipid phosphatase that dephosphorylates PI(3,4,5)P3 to PI(4,5)P2 (23).

We found that ephrin-A1 stimulation of PC3 cells, and most other types of cancer cells examined, dramatically inhibits phosphorylation of Akt at both T308 and S473, besides inhibiting phosphorylation of the Erk1/2 MAP kinases (Fig. 5A,B). Stimulation of PC3 cells with ephrin-A1 decreases Akt and S6 kinase phosphorylation over a wide range of concentrations, and ephrin-A1 Fc concentrations much lower than the typically used 1 µg/ml are sufficient to produce a strong effect. Interestingly, low ephrin-A1 levels appear to have more persistent effects on both the increase in tyrosine phosphorylated EphA2 and the decrease in Akt phosphorylation (Fig. 5A and data not shown). This may be due to the fact that lower ephrin-A1 Fc concentrations do not cause as much EphA2 receptor degradation and is consistent with our findings that ephrin-A1 Fc inhibits PC3 cell 2D growth on tissue culture plates, ability to form foci, and 3D growth in Matrigel over a wide range of concentrations (Figs. 1-3). We also observed similar Akt inactivation by ephrin-A1 Fc in growth medium containing 10% serum and after starving the cells by overnight growth in 0.5% serum, suggesting that Akt inactivation by EphA2 is a robust effect that can occur under different growth conditions (Fig. 5C). Remarkably, quantitative Meso Scale analysis (http://www.meso-scale.com) showed that the extent of dephosphorylation induced by ephrin-A1 Fc is similar to that induced by the potent PI3 kinase inhibitor Wortmannin (Fig. 5D). As reported in the previous funding period, phosphorylation of TSC2 at the Akt target site (T1462) and S6 kinase phosphorylation at the mTOR target site (T389) were also reduced. We also verified that EphA2 activation is sufficient to downregulate the mTOR pathway by showing that stimulation with the YSA agonistic peptide, which only activates EphA2, also inhibits phosphorylation of Akt (not shown).

These findings are particularly exciting because inhibition of Akt and mTOR by ephrin-stimulated EphA2 further substantiates the notion that the Eph family of receptor tyrosine kinases has the unusual ability to inhibit oncogenic pathways, in contrast to other receptor tyrosine kinases, which typically activate PI3 kinase and Akt. In agreement with our findings, a recent publication reports reduction of Akt phosphorylation in fibroblasts expressing a constitutively active B-Raf mutant, which increases EphA2 expression (24). We have begun investigating the mechanisms used by EphA2 to inhibit the Akt-mTOR pathway. The data we have obtained so far suggest that the mechanisms by which EphA2 regulates Akt phosphorylation are not those anticipated to be most likely.

Task 5. Characterize the involvement of PI3 kinase and its upstream regulatory signaling molecules in Akt inactivation caused by ephrin-A1-dependent stimulation of EphA2.

EphA2 inhibits Akt in cells expressing constitutively active Ras proteins. The previously reported inactivation of H-, K- or N-Ras downstream of EphA2 (10) could cause PI3 kinase and Akt inactivation (Fig. 4). However, we found that ephrin-A1 stimulation of the MDA-MB-231 breast cancer cell line, which harbors a constitutively active K-Ras mutant allele (Sanger Cosmic website), inhibits Akt but not Erk phosphorylation (not shown). Furthermore, we

transfected wild-type H-Ras or the constitutively active H-Ras G12V mutant in PC3 cells. Although both wild-type and mutant H-Ras similarly increased basal Akt phosphorylation, neither blocked Akt dephosphorylation induced by ephrin-A1 Fc (Fig. 6A). For comparison, we also examined Erk phosphorylation levels. The constitutively active H-Ras G12V enhanced the basal levels of Erk phosphorylation in PC3 cells much more than wild-type H-Ras, and H-Ras G12V but not wild-type H-Ras blocked ephrin-A1-dependent Erk dephosphorylation (Fig. 6A). This indicates that inactivation of Ras GTPases downstream of EphA2 is sufficient to explain Erk but not Akt inactivation.

EphA2 signaling also inhibits R-Ras, a more distant Ras family member known to activate PI3 kinase but not the Erk MAP kinase pathway (25). We therefore also expressed constitutively active R-Ras G38VY66F together with low levels of HA-tagged Akt in PC3 cells to preferentially monitor Akt phosphorylation in the transfected cells (representing ~40% of the cells). Expression of constitutively active R-Ras increased basal Akt phosphorylation but only slightly reduced the ephrin-dependent decrease in Akt phosphorylation detected in whole lysates and in anti-HA antibody immunoprecipitates (Fig. 6B and data not shown). These results suggest that inactivation of H-Ras, N-Ras, K-Ras and R-Ras does not play a major role in the inactivation of Akt downstream of EphA2 in PC3 cells and that other pathways must be involved.

Involvement of integrins in EphA2-dependent Akt dephosphorylation. Interestingly, the activity of several integrins can increase Akt phosphorylation, for example through PI3 kinase activation downstream of Fak (focal adhesion kinase) (26) and ILK (integrin-linked kinase) (27,28). Indeed, we have found that Akt phosphorylation is dramatically increased in PC3 prostate cancer and WM793 melanoma cells upon attachment to fibronectin or vitronectin, respectively, and that ephrin-A1 stimulation decreases this phosphorylation (not shown). Therefore, previous reports that EphA2 inhibits integrin-mediated cell adhesion in PC3 cells and other cell types (14,29) suggest that EphA2 signaling may inhibit Akt phosphorylation indirectly, by decreasing integrin-dependent cell substrate adhesion. However, manganese treatment to prevent integrin inactivation only slightly reduced ephrin-A1-dependent Akt dephosphorylation in PC3 cells (Fig. 7A) and WM793 cells (not shown). The efficacy of the manganese treatment was confirmed by the observed inhibition of cell contraction. Treatment with the 9EG7 β1 antibody to reduce β1 integrin inactivation in PC3 and WM793 cells plated on fibronectin also partially inhibited cell contraction without any detectable effect on ephrin-A1-dependent Akt dephosphorylation (Fig. 7B and data not shown). These results suggest that integrin inactivation does not play a major role in Akt inhibition downstream of EphA2.

EphA2 inhibits Akt phosphorylation in cells expressing constitutively activated PI3 kinase. To determine whether EphA2 may regulate Akt by inhibiting PI3 kinase through Ras-independent pathways, we first used several cancer cell lines expressing mutant, constitutively active PI3 kinase. Ephrin-A1 Fc stimulation of these cell lines, including HT-29 colorectal cancer and SKOV3 ovarian cancer cells resulted in robust Akt and S6 kinase dephosphorylation (Fig. 8), suggesting that inhibition of PI3 kinase activity is not likely to be responsible for Akt dephosphorylation downstream of EphA2. We also expressed a prenylated form of the p110α subunit of PI3 kinase in PC3 cells. This form of PI3 kinase is constitutively active because the prenyl group mediates permanent membrane association of this catalytic subunit. To preferentially monitor Akt phosphorylation in the transfected cells we co-expressed low levels of HA-tagged wild-type Akt (Fig. 9). As expected, we observed enhanced Akt phosphorylation in cells transfected with both Akt and constitutively active PI3 kinase, compared to cells transfected only with Akt. Akt phosphorylation in cells co-expressing constitutively active PI3 kinase was only slightly decreased by treatment with 0.1 µg/ml ephrin-A1 Fc (not shown). However, treatment with 1 µg/ml ephrin-A1 Fc substantially reduced Akt phosphorylation, albeit less than in cells transfected only with Akt (Fig. 9). These data suggest that inactivation of PI3 kinase might contribute to Akt inactivation downstream of EphA2, but that other pathways are

also involved. Alternatively, the high levels of Akt phosphorylation brought about by transfection of constitutively active PI3 kinase together with Akt can only be overcome by high levels of stimulation of EphA2-dependent pathways that do not involve PI3 kinase inactivation. To conclusively resolve whether PI3 kinase inhibition plays a role in Akt dephosphorylation downstream of EphA2, it will be important to determine whether EphA2 inhibits PI3 kinase activity in PC3 cells. However, in endothelial cells and MDA-MB-231 cells ephrin-induced activation of EphA2 has been shown to activate rather than inhibit PI3 kinase (30,31). Therefore, a mechanism involving inhibition of PI3 kinase downstream of EphA2 appears to be very unlikely.

EphA2 inhibits Akt phosphorylation in cells expressing constitutively activated Akt. We found that ephrin-A1 treatment also decreased phosphorylation of myristoylated Akt, which is constitutively active due to its constitutive membrane localization. This suggests that signaling events occurring downstream of PI3 kinase play an important role in Akt dephosphorylation (Fig. 10).

Task 6. Characterize the involvement of PI3 kinase-independent pathways in Akt inactivation caused by ephrin-A1-dependent stimulation of EphA2 in PC3 cells.

Involvement of the Ship2 lipid phosphatase in EphA2-dependent Akt inactivation. We found that ephrin-A1 stimulation decreases Akt and S6 kinase phosphorylation not only in PC3 prostate cancer cells but also in WM793, LU1205 and UACC903 cells, all of which lack functional Pten (23,32). This indicates that EphA2 does not decrease PIP3 levels by activating Pten. However, another PIP3 lipid phosphatase expressed in these cell lines, Ship2, has been proposed to functionally compensate for the loss of Pten (23). Furthermore, recent data show that EphA2 can promote Ship2 lipid phosphatase activity (31). We therefore investigated whether enhanced Ship2 activity may be responsible for Akt dephosphorylation in cells treated with ephrin-A1. In the previous funding period we prepared the SAM domain of EphA2 as an EGFP fusion protein. Transfection of this protein to inhibit EphA2-Ship2 interaction did not impair the ability of EphA2 to inactivate Akt, suggesting that Ship2 may not play an important role in this effect (not shown). Confirming this result, we also found that Ship2 downregulation by siRNA interference in PC3 cells only slightly decreased ephrin-A1-dependent dephosphorylation of Akt (Fig. 11), suggesting that regulation of Ship2 lipid phosphatase activity by EphA2 does not contribute in a major way to Akt inhibition. However, Ship2 knock-down did increase the basal level of Akt phosphorylation, indicating that Ship2 can indeed regulate Akt phosphorylation in PC3 cells (Fig. 11).

Involvement of phosphatases in Akt dephosphorylation downstream of EphA2. A likely possibility appears to be that EphA2, when stimulated by ephrin-A1, activates a serine-threonine phosphatase that can dephosphorylate Akt. The involvement of a phosphatase would be consistent with the rapid and profound effect of ephrin-A1 stimulation on Akt dephosphorylation, which can be detected in PC3 cells within 2 min (not shown). A search of the literature has uncovered several candidate phosphatases, including PHLPP1 and PHLPP2, PP2A, PP1, and PP2B (also known as calcineurin). So far, we have conclusively shown that PHLPP1 and PHLPP2, two widely expressed phosphatases that target S473 of Akt (33,34), do not play a major role. siRNA-mediated knockdown of these phosphatases individually or in combination in PC3 cells did not prevent EphA2-dependent Akt dephosphorylation (Fig. 12 and data not shown). Investigation of other phosphatases is one of the goals for the next year.

KEY RESEARCH ACCOMPLISHMENTS

- Demonstrated that ephrin-A1 Fc stimulation of PC3 cells inhibits 2D growth on tissue culture plates

- Demonstrated that ephrin-A1 Fc stimulation of PC3 cells inhibits 3D growth in focus formation assays
- Demonstrated that ephrin-A1 Fc stimulation of PC3 cells inhibits spheroid growth in Matrigel
- Demonstrated that ephrin-A1 Fc treatment causes PC3 prostate cancer cell contraction (retraction of the cell periphery and rounding) by inhibiting integrin-mediated cell substrate attachment
- Demonstrated that EphA2 activation by even low concentrations of ephrin-A1 Fc is sufficient to induce Akt dephosphorylation in PC3 cells
- Demonstrated that the extent of Akt dephosphorylation induced by ephrin-A1 Fc in PC3 cells is similar to that induced by the potent PI3 kinase inhibitor Wortmannin
- Demonstrated that inactivation of H-, K-, N- and R-Ras is not critical for Akt dephosphorylation downstream of EphA2 in PC3 cells
- Demonstrated that activation of the lipid phosphatase Ship2 is not critical for Akt dephosphorylation downstream of EphA2 in PC3 cells
- Demonstrated that inactivation of integrin-mediated adhesion is not critical for Akt dephosphorylation downstream of EphA2 in PC3 cells
- Demonstrated that possible PI3 kinase inactivation is not critical for Akt dephosphorylation downstream of EphA2 in PC3 cells
- Demonstrated that the constitutively active, membrane-bound myrAkt can be dephosphorylated downstream of EphA2 in PC3 cells
- Demonstrated that the PHLPP Akt phosphatases are not required for Akt dephosphorylation downstream of EphA2 in PC3 cells

REPORTABLE OUTCOMES

Abstracts:

Noberini R, Koolpe M, Peddibhotla S, Dahl R, Su Y, Roth GP, Cosford NDP, Pasquale EB (2008). Small molecules that selectively inhibit ephrin binding to EphA receptors. In "**Eph/Ephrins and Cancer Meeting, Wake Forest University**". Abstract poster #13, p. 12.

Original articles:

Noberini R, Koolpe M, Peddibhotla S, Dahl R, Su Y, Cosford NDP, Roth GP, Pasquale EB (2008). Small molecules can selectively inhibit ephrin binding to the EphA4 and EphA2 receptors. **J. Biol. Chem.** 283:29461-29472.

Review articles:

Pasquale EB (2008). Eph-ephrin bidirectional signaling in physiology and disease. **Cell** 133:38-52.

Research opportunities applied for:

Some of the data obtained with support from this grant have been included in the preliminary studies supporting an application for an NIH R01 grant, which was submitted for the Nov 5, 2008 deadline.

The reviewer of the 2006-2007 progress report noted that the following abstract and review articles, which were listed in the “Reportable oucomes” section of the report, should have been appended to the report. We are therefore enclosing them with the current report.

Abstracts:

Pasquale EB, Roselli S, Valencia F, Noren NK (2007). Tumor suppressor activity of the EphB2 receptor in prostate cancer. In “**Proceedings of IMPaCT Meeting, Atlanta**”. Abstract #P27-23, p. 263.

Review articles:

Noren NK, Pasquale EB (2007). Paradoxes of the EphB4 receptor in cancer. **Cancer Res.** 67:3994-3997.

Pasquale EB (2007). Eph receptors and ephrins. In “**Modern Concepts in Angiogenesis**”. Eds. M Simons and G Rubanyi, Imperial College Press, London, Chapter 18, pp. 27-66.

CONCLUSIONS

The work we have performed in the past 3 years under the umbrella of this project has confirmed our hypothesis that some Eph receptors can inhibit two oncogenic pathways that are critical for prostate cancer progression, the Ras-MAP kinase pathway and the Akt-mTOR pathway. Furthermore, they inhibit integrin-mediated adhesion. These effects support a functional role for the Eph receptors as tumor suppressor in prostate cancer when their signaling ability is activated by ephrin ligand stimulation. In addition to EphB2, we and others have gathered substantial evidence that the EphA2 receptor also has tumor suppressor activity in prostate cancer. The EphA2-dependent inhibition of Akt activity that we have uncovered, for example, can overcome the effects of mutations that promote tumor progression in prostate cancer, such as loss of the Pten phosphatase and constitutive activation of oncogenes such as PI3 kinase. Thus, therapeutic activation of EphA2 receptor signaling with ephrins or available activating antibodies (35) offers promise for anti-prostate cancer therapies. Clearly, the tumor suppressor activities of the Eph receptors in prostate cancer represent an important area of investigation that will help understand the pathogenesis of this disease and guide the design of novel diagnostic and therapeutic strategies.

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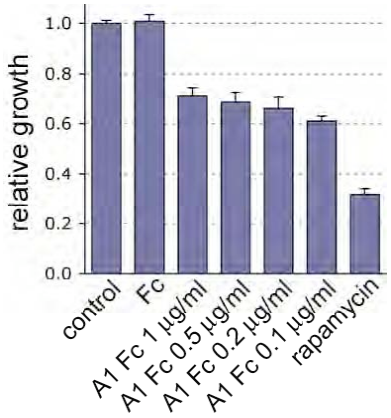

Fig. 1. EphA2 activation by ephrin-A1 inhibits the 2D growth of PC3 prostate cancer cell on tissue culture plates. Cells were grown in medium containing 10% serum and the indicated concentrations of Fc or ephrin-A1 Fc, or 100 nM rapamycin, and counted after 3 days. The histogram shows average relative cell growth ± SEM from triplicate measurements.

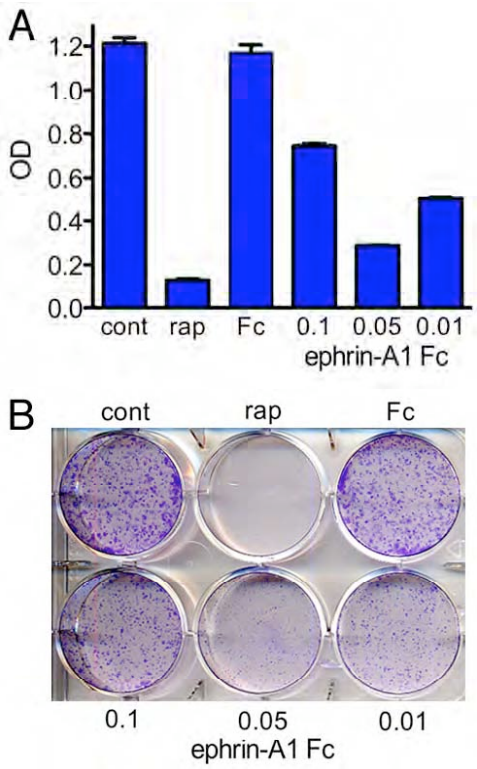

Fig. 2 EphA2 activation by ephrin-A1 inhibits PC3 prostate cancer cell growth in focus formation assays. Cells plated at low density were grown in medium containing 10% serum and the indicated concentrations of ephrin-A1 Fc (in µg/ml), control Fc (0.1 µg/ml), or 100 nM rapamycin. After 11 days the cells were stained with crystal violet and solubiilzed, and the absorbance at 570 nm was determined. The histogram shows average relative cell growth ± SEM from duplicate wells.

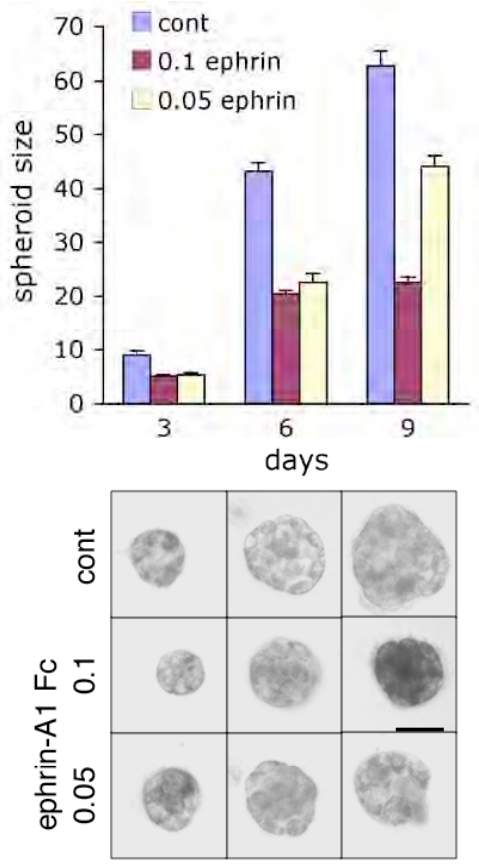

Fig. 3 EphA2 activation by ephrin-A1 inhibits the 3D growth of PC3 prostate cancer cells as spheroids. PC3 cells plated at low density in Matrigel were grown in medium containing 10% serum and the indicated concentrations of ephrin-A1 Fc (in µg/ml), control Fc (0.1 µg/ml), or 100 nM rapamycin. After 3, 6, or 9 days the spheroids were photographed and measured. The histogram shows average size in mm^3, calculated as (d max x d min^2 x π)/6 ± SEM (d = diameter). Scale bar = 50 µM.

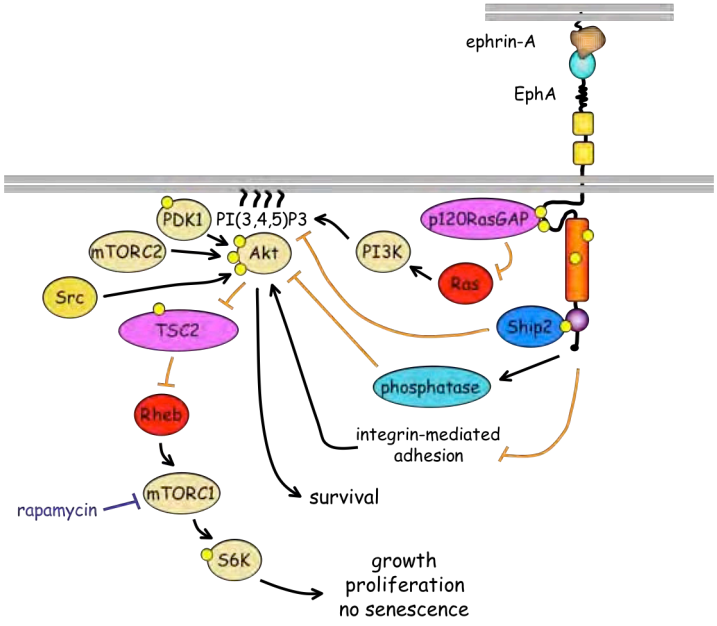

Fig. 4. Possible pathways leading to Akt inactivation downstream of the EphA2 receptor. Orange arrows indicate inhibition. Yellow circles indicate phosphorylation sites.

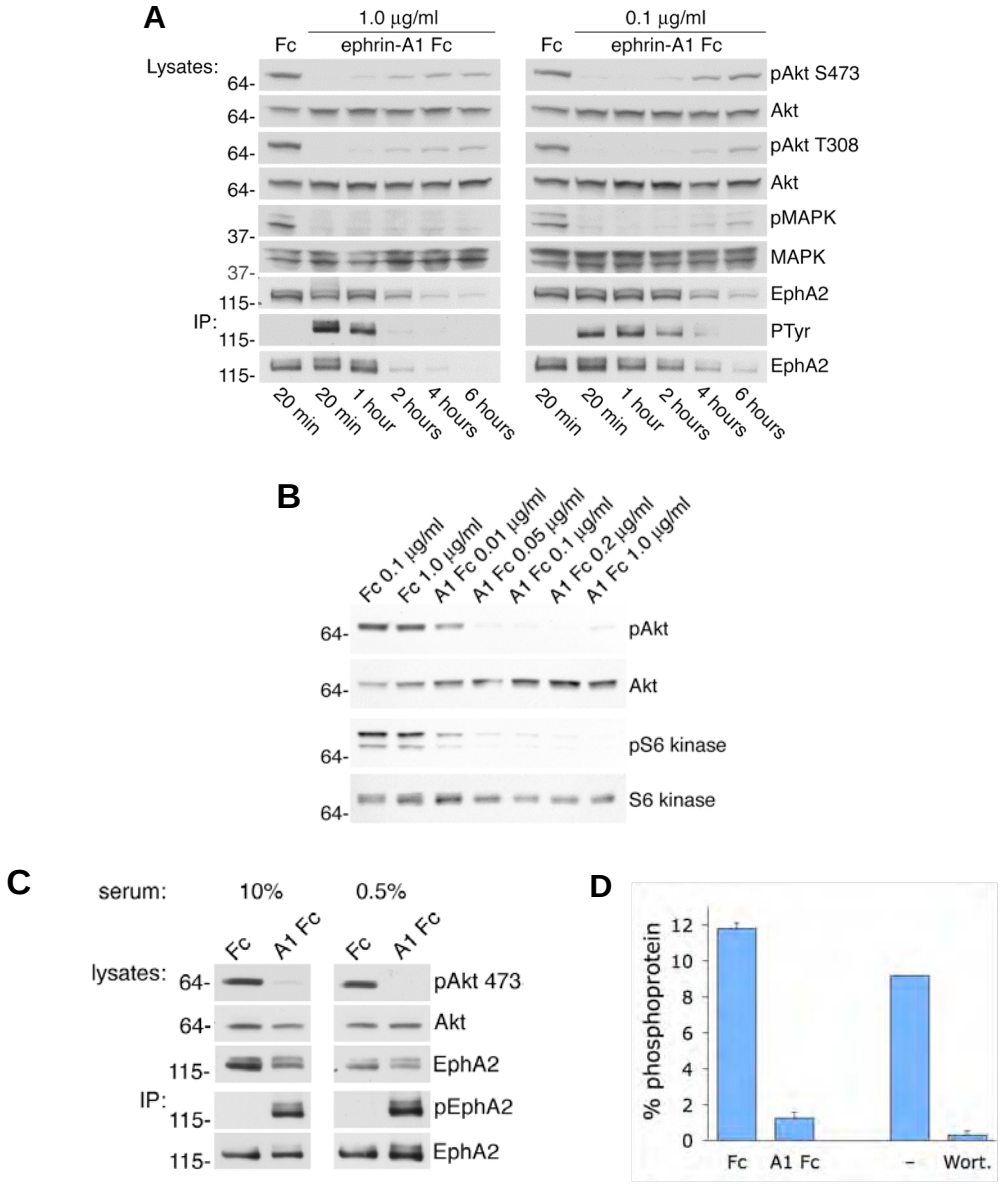

Fig. 5. EphA2 inhibits the Akt-mTOR and Erk-MAP kinase pathways in PC3 prostate cancer cells. (**A**) Cells grown in 10% serum were stimulated with the indicated concentrations ephrin-A1 Fc or Fc as a contro for the indicated times. Cell lysates were probed with antibodies to phosphorylated Akt and Erk1/2 MAP kinases and reprobed for the corresponding total proteins. EphA2 immunoprecipitates (IP) were probed for phosphotyrosine (PTyr) and reprobed for EphA2. EphA2 levels are more prominently downregulated at the higher concentrations of ephrin-A1 Fc. (**B**) Cells grown in 10% serum were stimulated for 20 min with the indicated concentrations of ephrin-A1 Fc or Fc as a control. Lysates were probed for phosphorylated Akt and S6 kinase and reprobed for the corresponding total protein. Even low ephrin concentrations substantially inhibit Akt phosphorylation. **(C)** Cells grown in 10% serum or starved overnight in 0.5% serum were stimulated for 20 min with 0.1 µg/ml ephrin-A1 Fc or Fc as a control. Lysates and EphA2 immunoprecipitates (IP) were probed with the indicated antibodies (pEphA2 indicates that anti-phosphotyrosine antibodies were used to detect autophosphorylated EphA2). Ephrin-A1 stimulation inhibits Akt phosphorylation in normal medium as well as in low serum-containing medium. (**D**) The % of Akt phosphorylated at S473 was measured in cells stimulated for 30 min with 1 µg/ml ephrin-A1 Fc or Fc as a control and in cells treated with 20 nM Wortmannin for 30 min or left untreated (–) using the Meso Scale technology. Ephrin-A1 decreases Akt phosphorylation similarly to the potent PI3 kinase inhibitor Wortmannin. The histogram shows averages ± SD from triplicate measurements for ephrin-A1 and duplicate measurements for Wortmannin.

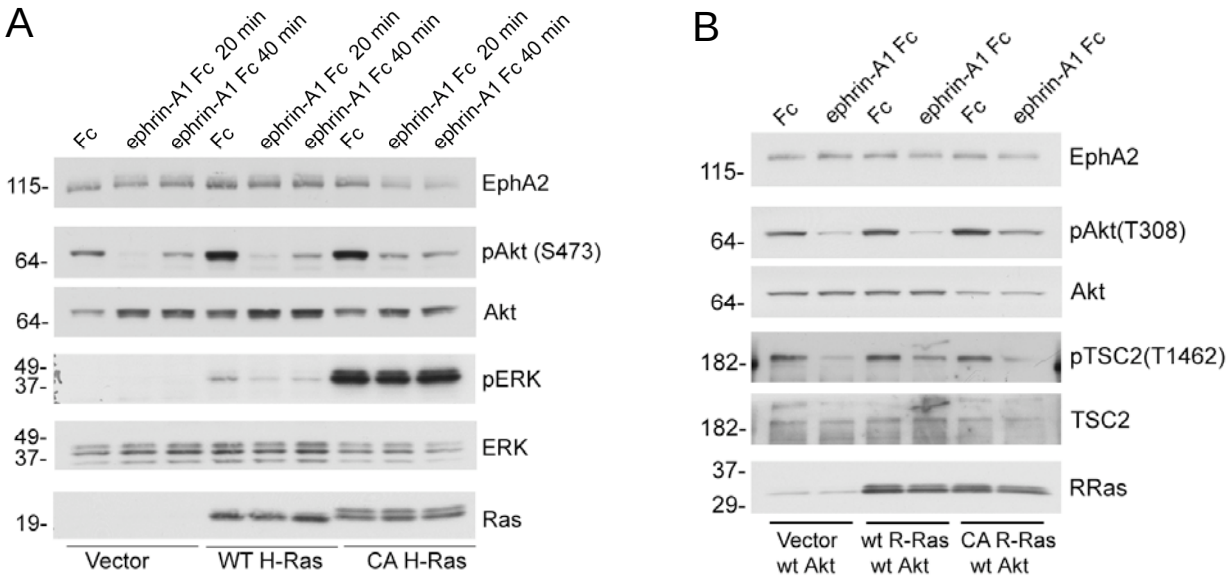

Fig. 6. EphA2 causes Akt dephosphorylation independently of H-Ras and R-Ras. (**A**) PC3 cells transfected with vector control, wild-type H-Ras, or constitutively active H-Ras G12V were grown in 10% serum and stimulated with 0.1 µg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies. (**B**) PC3 cells transfected with vector control, wild-type R-Ras, or constitutively active R-Ras G38VY66F were grown in 10% serum and stimulated with 0.1 µg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies.

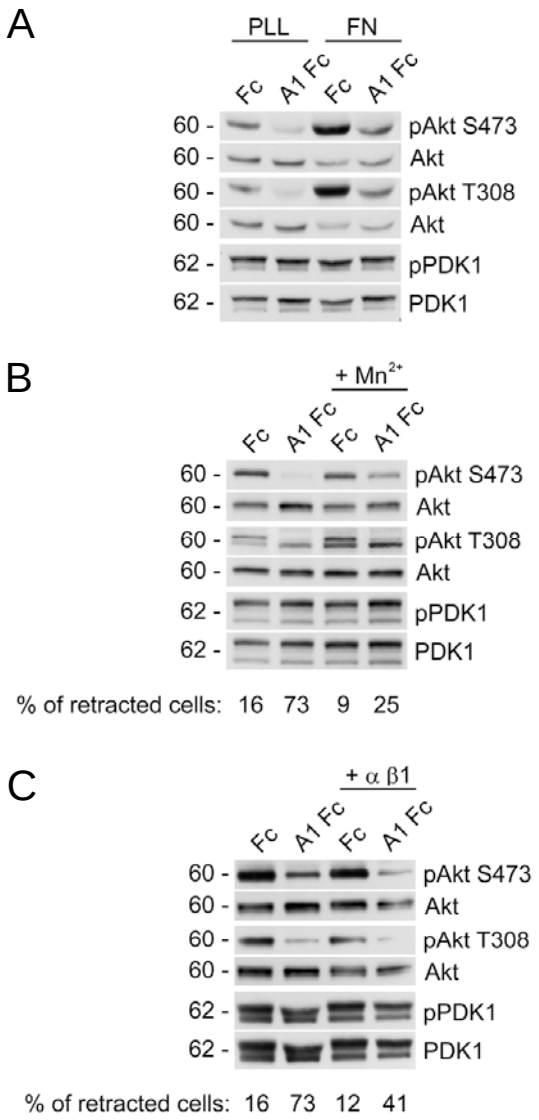

Fig. 7. EphA2 causes Akt dephosphorylation independently of integrin-mediated adhesion. (A) PC3 cells were plated on the β1-integrin ligand fibronectin (FN) or polylysine (PLL) as a control. They were then treated with 2 μg/ml ephrin-A1 Fc or Fc as a control. Blots were probed with the indicated antibodies, showing that integrin-mediated adhesion promotes Akt phosphorylation. **(B)** PC3 cells plated on tissue culture plates were treated with Mn^{2+} to maintain integrin activation even in the presence of ephrin-A1 Fc, or left untreated. Blots were probed with the indicated antibodies, showing that Mn^{2+} treatment does not prevent Akt dephosphorylation, although it successfully prevents retraction of the cell periphery. **(C)** PC3 cells plated on a fibronectin substrate were treated with an integrin activating antibody (a β1) to maintain β1 integrin activation even in the presence of ephrin-A1 Fc, or left untreated. Blots were probed with the indicated antibodies. Treatment with the integrin-activating antibody partially prevented retraction of the cell periphery, but did not prevent Akt dephosphorylation. Ephrin stimulation did not have any effect on the phosphorylation of PDK1, the kinase that phosphorylates T308 of Akt, in this or any other experiment.

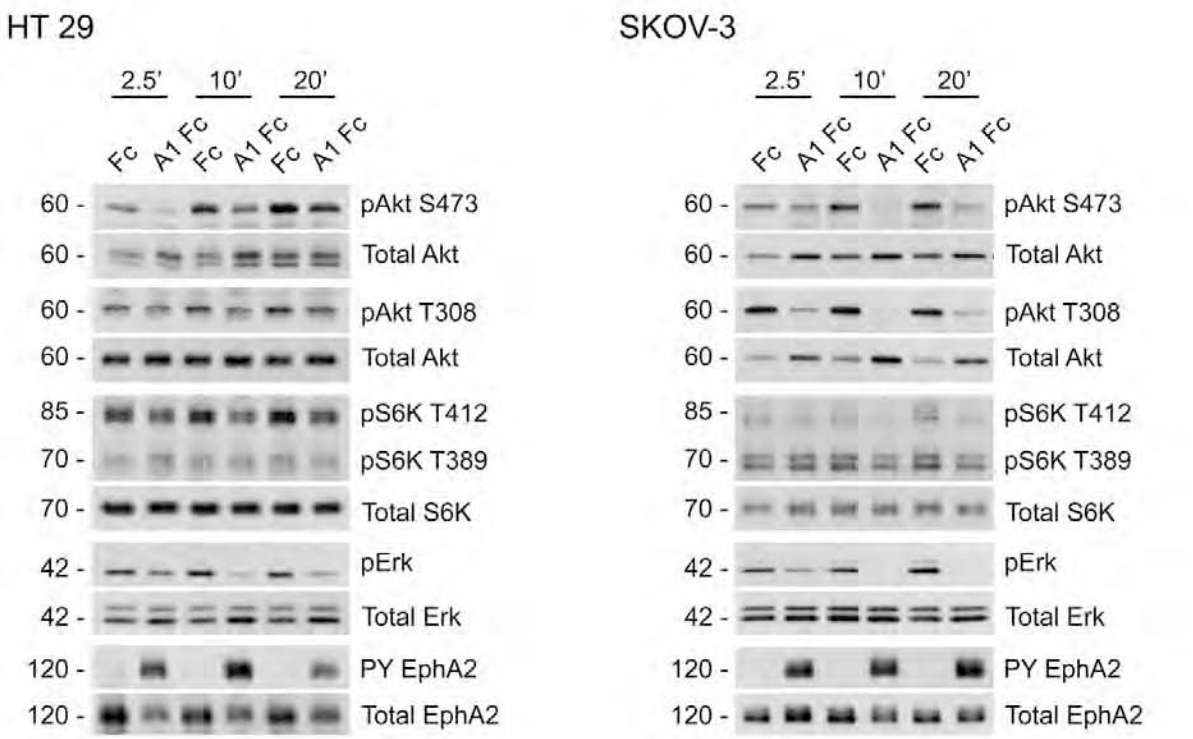

Fig. 8. EphA2 causes Akt dephosphorylation in cells expressing constitutively active PI3 kinase. HT-29 colorectal cancer cells and SKOV-3 ovarian cancer cells were stimulated with 2 µg/ml ephrin-A1 Fc for the indicated times in minutes. Lysates were probed with the indicated antibodies. For PY EphA2, anti-phosphotyrosine antibodies were used to detect autophosphorylated EphA2.

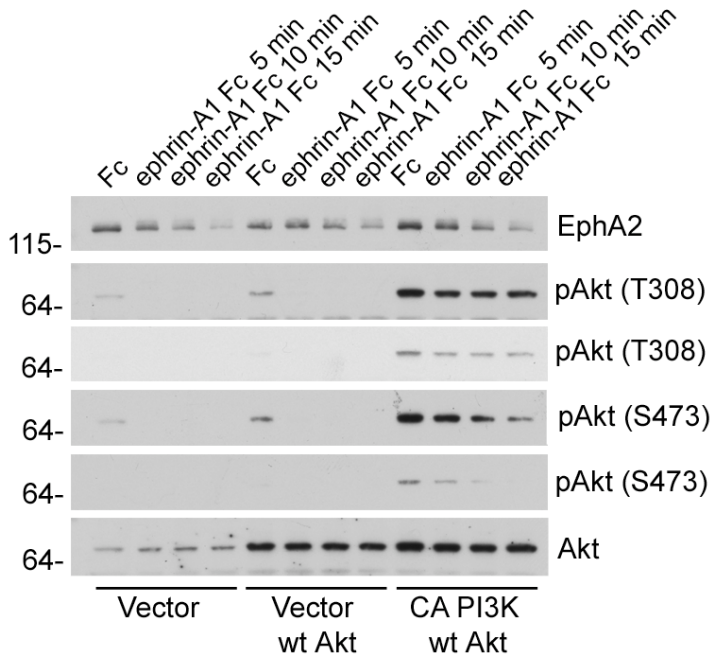

Fig. 9. EphA2 causes Akt dephosphorylation in cells transfected with constitutively active PI3 kinase and PIP3 regulation. Cells were transfected with vector control, wild-type Akt1, and wild-type Akt1 together with the constitutively active prenylated p110α subunit of PI3 kinase at a 1:4 Akt to PI3 kinase ratio. Cells were then stimulated with 1 µg/ml ephrin-A1 Fc. Lysates were probed with the indicated antibodies and long and short exposures are shown for the phosphoAkt blots.

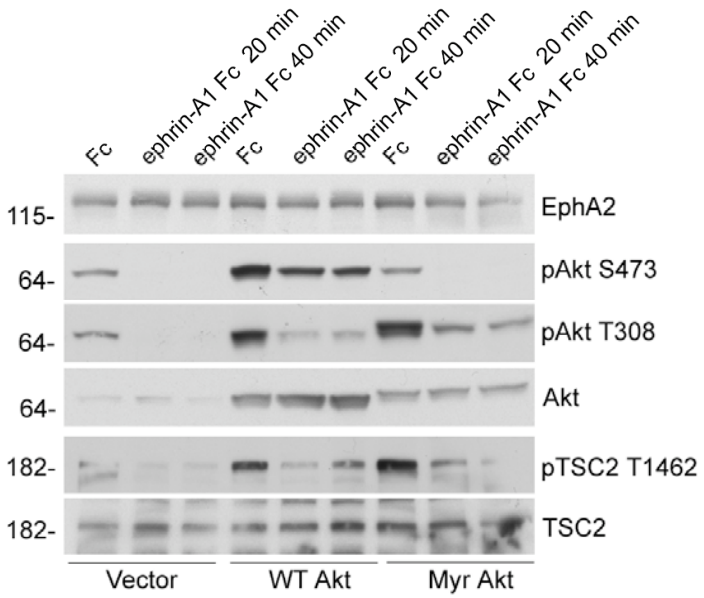

Fig. 10. EphA2 causes dephosphorylation of constitutively active, membrane-targeted Akt. Cells were transfected with vector control, wild-type Akt1, and myristoylated Akt1. Cells were then stimulated with 0.1 µg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies.

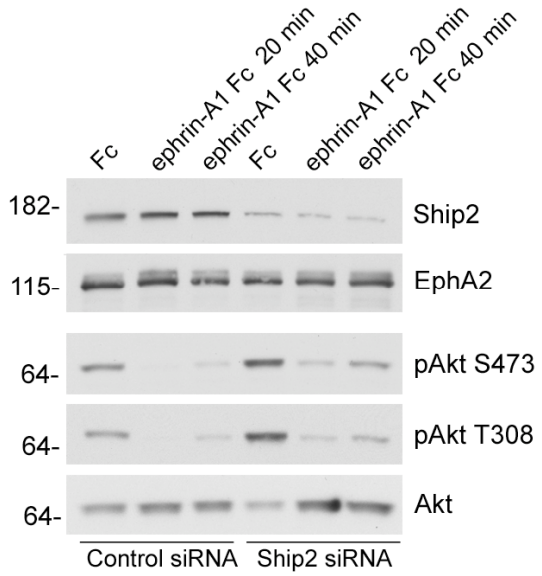

Fig. 11. EphA2 causes Akt dephosphorylation independently of the Ship2 lipid phosphatase. Cells were transfected with control siRNA or Ship2 siRNAs and stimulated with 0.1 µg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies.

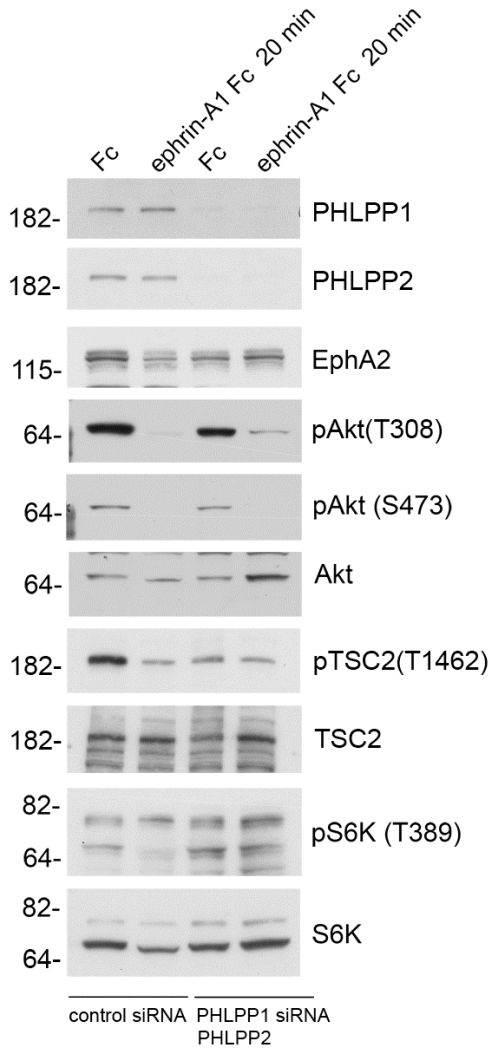

Fig. 12. EphA2 causes Akt dephosphorylation independently of PHLPP phosphatases. Cells were transfected with control siRNA or a combination of siRNAs for PHLPP1 and PHLPP2 phosphatases and stimulated with 0.1 µg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies.

Statement of Work - Revised

PI: Elena B. Pasquale
Title: Tumor Suppressor Activity of the EphB2 Receptor in Prostate Cancer

Aim 1. Determine whether the growth-suppressing activity of EphB2 and other Eph receptors in prostate cancer cells depends on activation by ephrin ligands

Task 1. Screen prostate cancer cell lines by immunoblotting and immunoprecipitation to determine expression and activation of selected Eph receptors and ephrins.

Task 2. Prepare pIRES-EGFP constructs for human EphB2 wild-type and kinase inactive mutant by subcloning and site-directed mutagenesis.

Task 3. Determine the effects of transiently transfected EphB2 and other ligand-activated Eph receptors on prostate cancer cell growth.

Aim 2. Characterize ephrin-dependent tumor suppressor signaling pathways activated downstream of EphA2 and EphB2 in prostate cancer cells

Task 4. Identify tumor suppressor signaling pathways stimulated by ephrins in prostate cancer cells.

Task 5. Characterize the involvement of PI3 kinase and its upstream regulatory signaling molecules in Akt inactivation caused by ephrin-A1-dependent stimulation of EphA2.

Task 6. Characterize the involvement of PI3 kinase-independent pathways in Akt inactivation caused by ephrin-A1-dependent stimulation of EphA2 in PC3 cells.

Task 7. Determine the involvement of Akt inactivation versus Erk1/2 MAP kinase inactivation in the ephrin-A1-dependent inhibition of prostate cancer cell growth.

Task 8. Assess whether ephrin-B-induced activation of EphB2 and other EphB receptors causes Akt inactivation in PC3 cells through the same signaling mechanisms used by EphA2.

Task 9. Determine whether EphB2 transiently transfected in DU145 cells causes Akt inactivation and whether this effect depends on ephrin binding.

List of Appendices

- Noberini R, Koolpe M, Peddibhotla S, Dahl R, Su Y, Roth GP, Cosford NDP, Pasquale EB (2008). Small molecules that selectively inhibit ephrin binding to EphA receptors. In "**Eph/Ephrins and Cancer Meeting, Wake Forest University**". Abstract poster #13, p. 12.

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Small molecules that selectively inhibit ephrin binding to EphA receptors

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Eph receptors and their ligands, the ephrins, are involved in a multitude of physiological and pathological processes, both during development and in adult tissues. Although agents modulating Eph-ephrin interaction could have wide application as research tools and as therapeutic agents, no small molecule inhibitors of Eph receptor-ephrin interaction have been identified so far. EphA4, which can bind all the ephrin ligands and is the most promiscuous of the Eph receptors, has been linked to diverse pathologies, such as inhibition of nerve regeneration after spinal cord injury and certain types of cancer. We have used a high throughput screening approach to identify small molecules able to inhibit ligand binding to EphA4. This led to the identification of two isomeric chemical compounds that selectively inhibit ephrin binding to only two receptors, EphA4 and EphA2. The compounds behave like competitive inhibitors, suggesting that they target the high affinity ligand-binding site of the receptors. Furthermore, they inhibit EphA4 and EphA2 phosphorylation in cells, albeit at high concentrations, without affecting cell viability or the phoshorylation of other receptor tyrosine kinases. The two compounds also counteract EphA4-mediated growth cone collapse in retinal neuron and EphA2-mediated morphological changes in prostate cancer cells. Therefore, modified compounds with increased affinities could be used as leads for the development of pharmaceuticals to treat pathologies involving EphA4 and EphA2.
- poster #13

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Small Molecules Can Selectively Inhibit Ephrin Binding to the EphA4 and EphA2 Receptors*[S]

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The erythropoietin-producing hepatocellular (Eph) family of receptor tyrosine kinases regulates a multitude of physiological and pathological processes. Despite the numerous possible research and therapeutic applications of agents capable of modulating Eph receptor function, no small molecule inhibitors targeting the extracellular domain of these receptors have been identified. We have performed a high throughput screen to search for small molecules that inhibit ligand binding to the extracellular domain of the EphA4 receptor. This yielded a 2,5-dimethylpyrrolyl benzoic acid derivative able to inhibit the interaction of EphA4 with a peptide ligand as well as the natural ephrin ligands. Evaluation of a series of analogs identified an isomer with similar inhibitory properties and other less potent compounds. The two isomeric compounds act as competitive inhibitors, suggesting that they target the high affinity ligand-binding pocket of EphA4 and inhibit ephrin-A5 binding to EphA4 with K_i values of 7 and 9 μM in enzyme-linked immunosorbent assays. Interestingly, despite the ability of each ephrin ligand to promiscuously bind many Eph receptors, the two compounds selectively target EphA4 and the closely related EphA2 receptor. The compounds also inhibit ephrin-induced phosphorylation of EphA4 and EphA2 in cells, without affecting cell viability or the phosphorylation of other receptor tyrosine kinases. Furthermore, the compounds inhibit EphA4-mediated growth cone collapse in retinal explants and EphA2-dependent retraction of the cell periphery in prostate cancer cells. These data demonstrate that the Eph receptor-ephrin interface can be targeted by inhibitory small molecules and suggest that the two compounds identified will be useful to discriminate the activities of EphA4 and EphA2 from those of other co-expressed Eph receptors that are activated by the same ephrin ligands. Furthermore, the newly identified inhibitors represent possible leads for the development of therapies to treat pathologies in which EphA4 and EphA2 are involved, including nerve injuries and cancer.

The Eph[2] receptors compose a large family of receptor tyrosine kinases that have been extensively studied for their roles in the developing and adult nervous system and in the developing cardiovascular system (1–6). In recent years the Eph receptors have also been implicated in many different physiological and pathological processes, including the regulation of insulin secretion, bone homeostasis, immune function, blood clotting, pathological forms of angiogenesis, and cancer (7). The ability to modulate the activities of this family of receptors is therefore of critical interest to gain a better understanding of their functions in the physiology of many organs and in various pathological conditions, as well as for medical therapy.

The Eph receptors exert their effects by interacting with ligands, the ephrins, which are also membrane-bound proteins. Eph receptor-ephrin interaction is mediated by two binding sites in the amino-terminal ephrin-binding domain of the receptor as follows: a high affinity site, which includes a hydrophobic cavity that accommodates a protruding loop of the ephrin (the G-H loop), and a separate low affinity site (8). A third molecular interface located in the adjacent cysteine-rich region of the receptor has also been described (9). Despite the presence of several binding interfaces, peptides that target the high affinity site are sufficient to inhibit Eph receptor-ephrin binding (10–12). Interestingly, unlike the ephrins whose binding is highly promiscuous, a number of the peptides that were identified by phage display selectively bind to only one or a few of the Eph receptors (10, 13, 14).

Other molecules that modulate Eph-ephrin interactions have also been identified, including antibodies and soluble forms of Eph receptors and ephrins extracellular domains (2, 15–17). Several small molecule inhibitors of the Eph receptor kinase domain have also been reported (18–21). These inhibitors occupy the ATP binding pocket of the receptors and are usually broad specificity inhibitors that target different families of tyrosine kinases (18, 19). Epigallocatechin gallate, a green tea derivative known to inhibit several tyrosine kinases, has also been shown to inhibit EphA receptor-mediated a human umbilical vein endothelial cell (HUVE)

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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[2] The abbreviations used are: Eph, erythropoietin-producing hepatocellular; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium; TNFα, tumor necrosis factor-α; PBS, phosphate-buffered saline; ANOVA, analysis of variance; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; AP, alkaline phosphatase; HUVE, human umbilical vein endothelial.

migration and capillary-like tube formation, but the mechanism of action of this molecule has not been elucidated (22). Although the size, polarity, and geometry of the high affinity ephrin-binding pocket of the Eph receptors suggest that it might accommodate the binding of a small molecular weight chemical compound (23), no such inhibitors have been identified so far for any of the Eph receptors.

The Eph receptors are subdivided in two classes, which in the human genome include nine EphA receptors, which preferentially bind the five ephrin-A ligands, and five EphB receptors, which preferentially bind the three ephrin-B ligands. Binding between receptors and ephrins of the same class is highly promiscuous, and few examples of inter-class binding have also been reported (24). In particular, EphA4 can bind both ephrin-A and ephrin-B ligands and represents the most promiscuous member of the Eph family. This peculiar feature of EphA4 makes its ephrin-binding pocket particularly interesting to target. Furthermore, besides being a well know regulator of neural connectivity during development and of synaptic function in the adult brain (25, 26), EphA4 has also been linked to several pathologies, which suggests that this receptor could be a promising new target for drug development. For example, EphA4 has been implicated in the inhibition of spinal cord regeneration after injury, by promoting the formation of the glial scar and inhibiting axon regrowth (27–29). In addition, EphA4 is expressed on the surface of human platelets, where it promotes thrombus stabilization (30). EphA4 has also been detected in different types of cancer cells (31–33) as well as in tumor endothelial cells (34, 35). Hence, modulation of EphA4-ephrin binding could be useful in the treatment of different pathological conditions.

In this study, we have used a high throughput screening approach to identify small molecular weight compounds that inhibit ligand binding to the EphA4 receptor. This screen identified two isomeric 2,5-dimethylpyrrolyl benzoic acid derivatives that selectively inhibit ephrin binding to EphA4 and EphA2 as well as the functions of these receptors in live cells.

EXPERIMENTAL PROCEDURES

Chemical Library Screening for EphA4 Inhibitors—A 96-well format *in vitro* assay was used for compound screening. Polystyrene high binding capacity plates (Corning Glass) were incubated for 18 h at room temperature with 2 μg/ml streptavidin (Pierce) diluted in borate buffer (0.1 M boric acid, 0.1 M sodium borate, pH 8.7), washed three times with binding buffer (Tris-buffered saline (TBS: 150 mM NaCl, 50 mM Tris-HCl, pH 7.5) with 1 mM $CaCl_2$ and 0.01% Tween 20), blocked with 0.5% bovine serum albumin in TBS for 1 h at room temperature, washed three times with binding buffer, and then coated by overnight incubation at 4 °C with 0.1 μM biotinylated KYL peptide (14) in binding buffer. Peptide-coated plates were washed five times with binding buffer, and compounds were added to the wells at a final concentration of 10 μg/ml in 1% dimethyl sulfoxide (DMSO) together with EphA4 alkaline phosphatase fusion protein (EphA4 AP) produced from transfected cells. Cell culture medium containing the secreted EphA4 AP was diluted 1:16 in binding buffer. The mixture was incubated for 3 h at room temperature on a plate shaker before washing five times with binding buffer and adding as the substrate 1 mg/ml *p*-nitrophenyl phosphate (Pierce) in SEAP buffer (105 mM diethanolamine, 0.5 mM $MgCl_2$, pH 9.8). After 1 h at room temperature the reaction was stopped by adding 2 N NaOH, and the absorbance at 405 nm was measured using an ELISA plate reader. Alkaline phosphatase activity from wells where AP was added instead of EphA4 AP was subtracted as background. The inhibitory activity of the compounds was calculated by dividing the absorbance observed in the presence of compound and the absorbance from wells where no compound was added. Compounds with inhibitory activity higher than 50% were considered hits. The inhibitory activity of the hits was confirmed by repeating the assay.

ELISAs and K_i Determination—Protein A-coated wells (Pierce) were used to immobilize ephrin Fc fusion proteins (R & D Systems, Minneapolis, MN). Compounds at different concentrations were incubated in the wells with EphA4 AP (36) or EphA2 AP (13) for 3 h. Alternatively, Eph receptor Fc fusion proteins were immobilized on protein A-coated wells, and ephrin-A5 AP (37) or ephrin-B2 AP (GeneHunter, Nashville, TN) was added with the compounds. The amount of bound AP fusion protein was quantified using *p*-nitrophenyl phosphate as the substrate. Alkaline phosphatase activity from wells with Fc only was subtracted as background.

To confirm that the binding of the compounds to EphA4 was reversible, the compounds were removed, and the wells were incubated in binding buffer for 3 h before washing and incubating with ephrin AP fusion proteins. Under these conditions, no inhibition of ephrin binding was observed, as expected for reversible inhibitors. Further control experiments verified that the compounds do not inhibit the activity of alkaline phosphatase in solution and also do not inhibit binding of EphA4 AP to an anti-EphA4 antibody (R & D Systems, Minneapolis, MN) immobilized to protein G-coated plates (Pierce), ruling out nonspecific inhibitory effects.

To calculate the inhibition constant (K_i) values, the binding of ephrin-A5 AP to EphA4 Fc immobilized on protein A-coated wells was measured in the absence and in the presence of the compounds at different concentrations. Each data set was fitted to the Michaelis-Menten equation: $B = B_{max}[S]/(K_D + [S])$, where [S] is the concentration of ephrin AP fusion protein, and K_D is the dissociation constant observed in the absence or in the presence of the compound, using nonlinear regression and the program Prism (GraphPad Software Inc.). To evaluate whether the inhibition is competitive, noncompetitive, or uncompetitive, the K_D and B_{max} values were determined at different compound concentrations. The K_i was obtained from the linear regression plot of K_D/B_{max} as a function of the concentration of the inhibitor according to the following: $K_D/B_{max} = (K_D[S])/(K_i B_{max}) + K_D/B_{max}$. Alternatively, K_i values were obtained from the dose-response curves, using the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [S]/K_D)$ (38). Ephrin-A5 AP concentrations were calculated from alkaline phosphatase activity (39).

Chemical Synthesis—Compounds were purchased from ChemBridge; with the exception of compound **29** (Matrix Scientific, Columbia, SC), compounds **14** and **33** (Sigma), com-

pound **21** (Key Organics, Cornwall, UK), compounds **8** and **39** (ChemDiv, San Diego), and compounds **3–5**, **7**, **19**, **22**, **26**, **27**, **37**, **40–42**, **47**, **54,** and **55**, which were synthesized as described below. Furthermore, as a control compound **1** was also synthesized as well as purchased from InterBioScreen (Moscow, Russia).

For the synthesis of compounds **1**, **26, 27, 37, 39, 41, 42,** and **54**, a 15-ml glass pressure vessel was charged with the appropriate aniline (1.0 mmol), 2,5-hexanedione (1.2 mmol), *p*-toluenesulfonic acid (0.2 mmol), and toluene (5.0 ml). The mixture was stirred and heated under reflux for 24 h. After evaporation of the toluene, the crude product was purified first by flash chromatography (ethyl acetate/hexanes) and then by reverse phase chromatography. The final products were lyophilized to give solids in yields ranging from 47 to 82%. Final product purities of greater than 95% were confirmed by ^{1}H NMR or liquid chromatography/mass spectrometry.

For the synthesis of compounds **3, 4, 7,** and **19**, a 35-ml microwave tube was charged with the appropriate aniline (1.0 mmol), 2,5-hexanedione (1.2 mmol), *p*-toluenesulfonic acid (0.2 mmol), and ethanol (5.0 ml). The mixture was heated under microwave irradiation at 180 °C for 5 min. The solvent was then evaporated, and the residue was subjected to flash chromatography (0–15% ethyl acetate/hexanes or 0–10% methanol/dichloromethane) and then reverse phase chromatography if required. The final products were lyophilized to give solids in yields ranging from 30 to 80%. Final product purities of greater than 95% for compounds **4, 7,** and **19**, and greater than 80% for compound **3** were confirmed by ^{1}H NMR or liquid chromatography/mass spectrometry.

For the synthesis of compounds **5, 22, 40,** and **47**, the appropriate aryl halide (0.5 mmol) was mixed with 2,5-dimethylpyrrole (0.7 mmol), CuI (0.1 mmol), *N*-methylglycine (0.2 mmol), and potassium carbonate (1.5 mmol) in dimethylformamide (5.0 ml). The mixture was placed in a sealed glass vial and irradiated under microwave conditions at 200 °C for 20 min. The resulting mixture was cooled, filtered, and concentrated *in vacuo*. The resulting residue was dissolved in acetonitrile and purified via reverse phase chromatography. After lyophilization, the product pyrroles were furnished as solids with yields ranging from 26 to 57%. Final product purities of greater than 95% were confirmed by ^{1}H NMR or liquid chromatography/mass spectrometry. The identity and purity of all the synthesized compounds and compound **1** purchased from InterBioScreen was verified by liquid chromatography/mass spectrometry.

Measurement of Receptor Tyrosine Phosphorylation in Cells—HT22 neuronal cells, which endogenously express EphA4, are derived from immortalized mouse hippocampal neurons (40). COS7 cells, which endogenously express EphA2, EphB2, and the epidermal growth factor (EGF) receptor, were obtained from ATCC. Both cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) (Mediatech, Inc, Herndon, VA) with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT) and penicillin/streptomycin. For EphA4 immunoprecipitations, HT22 cells were serum-starved overnight in 0.5% FBS in DMEM and incubated for 15 min with the compounds or DMSO as a control. The cells were then stimulated with 0.5 μg/ml ephrin-A5 Fc, ephrin-A4 Fc, or Fc for 20 min in the continued presence of the compounds. After stimulation the cells were lysed in modified RIPA buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 20 mM Tris, 150 mM NaCl, 1 mM EDTA) containing 10 μM NaF, 1 μM sodium pervanadate, and protease inhibitors. Protein concentrations were calculated using the BCA protein assay kit (Pierce). Cell lysates were immunoprecipitated with 4 μg of anti-EphA4 antibody (41).

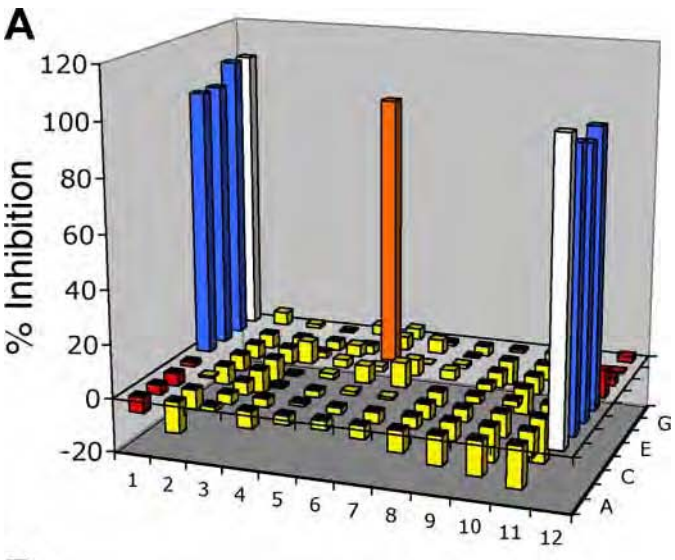
For EphA2 and EphB2 immunoprecipitations, serum-starved COS7 cells were stimulated with 0.1 μg/ml ephrin-A1 Fc or 0.5 μg/ml ephrin-B2 Fc, respectively. The cells were then lysed and incubated with 2 μg of anti-EphA2 antibody (Millipore-Upstate, Inc, Temecula, CA) or 7 μg of anti-EphB2 antibody made to a glutathione *S*-transferase fusion protein of the EphB2 carboxyl-terminal tail (42). To assess EGF receptor phosphorylation, COS7 cells were serum-starved overnight in 0.2% FBS in DMEM. The cells were preincubated with the compounds as described above and then stimulated for 15 min with 0.1 μM EGF. PC3 cells were grown in RPMI 1640 medium (Mediatech, Inc, Herndon, VA) with 10% FBS and penicillin/streptomycin. EphA2 was immunoprecipitated from PC3 cells as described above but after stimulation with 0.5 μg/ml ephrin-A1 Fc.

To assess inhibition of EphA2 phosphorylation in response to endothelial cell stimulation with tumor necrosis factor-α (TNFα), HUVE cells obtained from Cascade Biologics (Portland, OR) were grown in Medium 200 supplemented with low serum growth supplements (Cascade Biologics), 10% FBS, penicillin/streptomycin, and fungizone. The cells were serum-starved overnight in 2% FBS containing medium before adding 7 nM TNFα together with the compound or DMSO for 2 h.

Immunoprecipitates and lysates were probed by immunoblotting with anti-phosphotyrosine antibody (Millipore Inc., Temecula, CA) and reprobed with antibodies to the respective Eph receptors or anti-EGF receptor antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) followed by secondary peroxidase-conjugated antibodies (GE Healthcare). The EphA2 and EphA4 antibodies used for immunoblotting were from Invitrogen/Zymed Laboratories Inc.

MTT Assay—The cytotoxicity of the compounds was measured using the MTT colorimetric assay. Cells were seeded in 96-well plates and treated with compounds or DMSO starting 3, 2, or 1 day before they reached 100% confluency. For the assay, MTT (Sigma) was added at a final concentration of 0.5 μg/ml and incubated with the cells for 3 h. The resulting formazan crystals were then solubilized by addition of 100% DMSO. The absorbance in each well was measured at 570 nm using an ELISA plate reader. The results were expressed as the ratio of the absorbance of the cells treated with the compounds or left untreated.

Growth Cone Collapse Assay—Nasal retina explants from embryonic day 6 chicken embryos were cultured on coverslips coated with 200 μg/ml poly-L-lysine and 20 μg/ml laminin for 12–24 h in DMEM/F-12 culture medium containing 10% FBS and 0.4% methylcellulose. Three hours before adding the Fc fusion proteins, the medium was changed to DMEM/F-12 without methylcellulose. The explants were



B

Structure	MW	% Inhib. EphA4 KYL	IC_{50} (μM) EphA4 KYL	IC_{50} (μM) EphA4 ephrin-A5
1	231	99 99	3±0.5	13±1.3
6	229	65 70	14	>1000
8	245	81 90	25	>1000
10	229	58 66	56	>1000

FIGURE 1. **High throughput screening identifies small molecules that inhibit EphA4 ligand binding.** *A,* results from the screen showing the ELISA plate from which compound **1** was identified. *Orange*, well containing compound **1**; *yellow*, wells containing compounds from the library that are not inhibitory; *red*, control wells containing EphA4 AP; *blue*, control wells containing AP; and *white*, control wells containing only buffer. *B,* 2,5-dimethylpyrrolyl benzene derivatives identified in the high throughput screening for EphA4 inhibitors; the names of the compounds, indicated at the *left* of the structures, correspond to those in Fig. 4. The first value in the % inhibition (*% Inhib.*) column was obtained in the original screen with 10 μg/ml compound; the second value was obtained in a confirmatory repeat of the experiment. IC_{50} values were calculated by measuring binding of EphA4 AP to immobilized KYL peptide or ephrin-A5 AP to immobilized EphA4 Fc in the presence of different concentrations of the compounds.

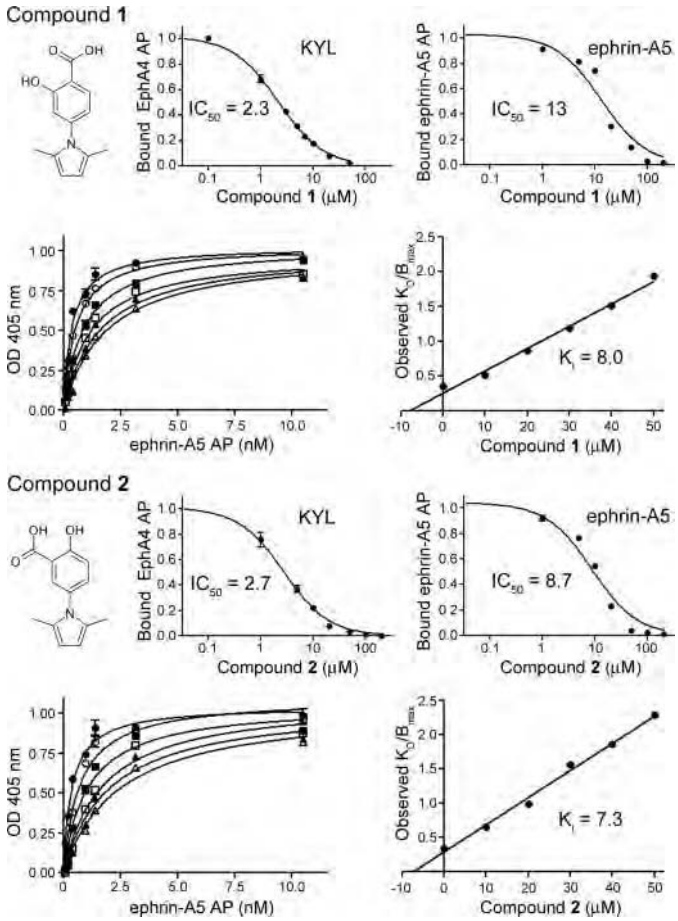


FIGURE 2. **Small molecules identified by high throughput screening inhibit ephrin-A5 binding to EphA4 in a competitive manner.** Compound **1** and compound **2** inhibit EphA4 AP binding to immobilized biotinylated KYL peptide and ephrin-A5 AP binding to immobilized EphA4 ectodomain fused to Fc in a concentration-dependent manner, as shown in the *two top panels* for each compound. The *bottom left panels* show the binding of ephrin-A5 AP to immobilized EphA4 Fc in the presence of different concentrations of each compound as follows: (●), 0 μM; (○), 10 μM; (■), 20 μM; (□), 30 μM; (▲), 40 μM; (Δ), 50 μM. These curves were used to calculate the dissociation constants (K_D) and maximal binding (B_{max}) used in the *bottom right panels* to determine K_i values. *Error bars* represent standard errors from triplicate measurements.

incubated for 15 min with the KYL peptide or the compounds and then stimulated for 30 min with 1 μg/ml preclustered ephrin-A5 Fc or Fc as a control. The cultures were fixed for 30 min in 4% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS), permeabilized in 0.1% Triton X-100 in PBS, and stained with rhodamine-conjugated phalloidin (Invitrogen). Cells were photographed under a fluorescence microscope, and growth cones were scored in a blinded manner as collapsed when no lamellipodia or filopodia were present at the tip of the neurite.

PC3 Cell Retraction Assay—PC3 cells were plated on glass coverslips, and after 17 h they were starved for 3 h in 0.5% FBS in DMEM and then incubated for 40 min with the compounds or DMSO, before stimulation for 10 min with 0.5 μg/ml ephrin-A1 Fc or Fc as a control. The cells were then fixed in 4% formaldehyde in PBS, permeabilized in 0.5% Triton X-100 in TBS, and stained with rhodamine-conjugated phalloidin (Invitrogen) and 4′,6-diamidino-2-phenylindole. Cells were

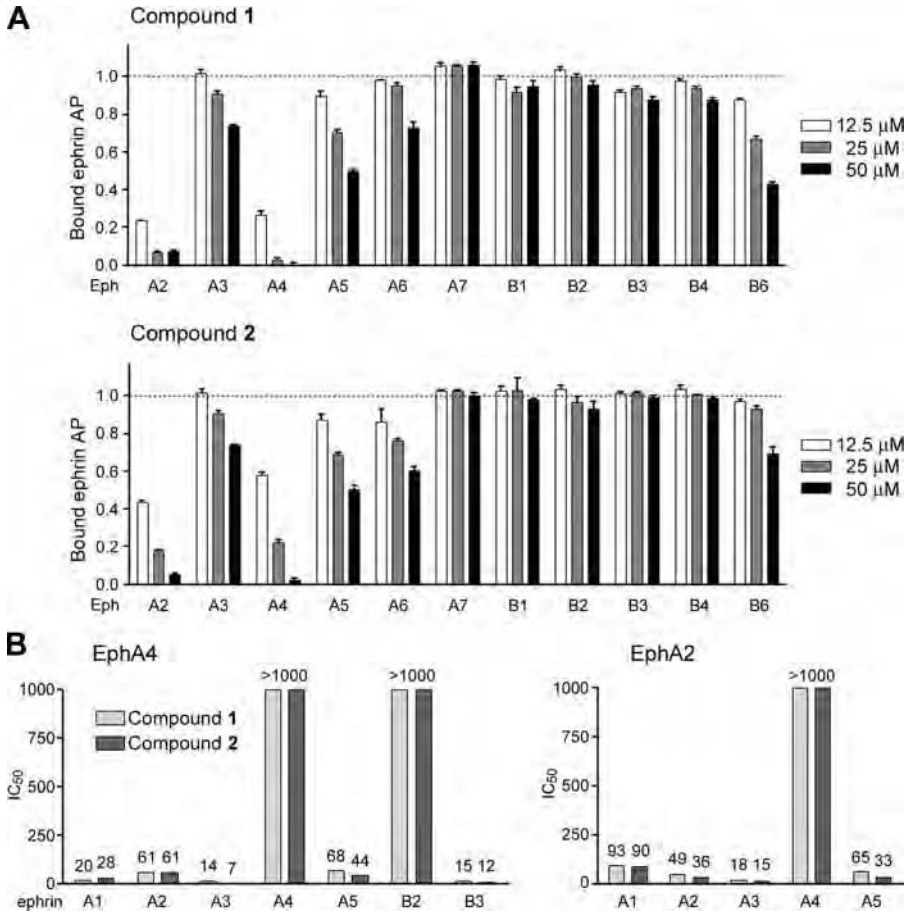


FIGURE 3. **Compounds 1 and 2 are selective in their inhibition of Eph receptor-ephrin interactions.** *A*, ephrin-A5 AP binding to immobilized EphA receptor Fc fusion proteins and ephrin-B2 AP binding to immobilized EphB receptor Fc fusion proteins were measured in the presence of the indicated concentrations of compounds **1** and **2**. The *histogram* shows the ratio of ephrin AP bound in the presence and in the absence of the compounds. *Error bars* represent standard errors from triplicate measurements. *B*, IC_{50} values for inhibition of EphA4 AP and EphA2 AP binding to the indicated immobilized ephrins by compounds **1** and **2**.

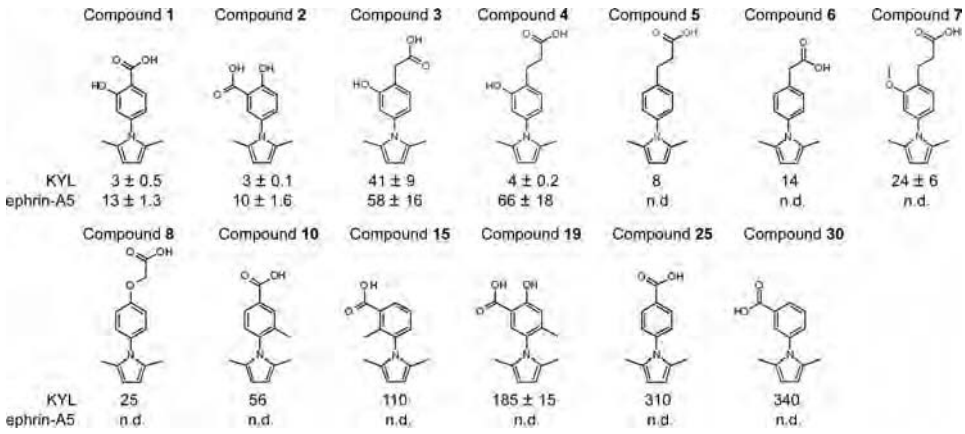


FIGURE 4. **Structure-activity relationship analysis of small molecules related to compounds 1 and 2.** Structures of some 2,5-dimethylpyrrolylbenzoic acid derivatives that were examined and their IC_{50} values (μM) for inhibition of EphA4 AP binding to the KYL peptide and ephrin-A5 AP binding to EphA4 Fc. Standard errors are indicated for IC_{50} values obtained from multiple experiments. The compounds are arranged in order of decreasing potency for inhibition of EphA4-ephrin-A5 binding or EphA4-KYL binding. Only compounds **1–4** were able to detectably inhibit EphA4-ephrin-A5 binding; *n.d.* indicates that inhibitory effects were not detectable. Additional compounds that were examined are shown in supplemental Fig. 1.

photographed under a fluorescence microscope, and cell area was measured in a blinded manner using ImageJ software (rsb.info.nih.gov). Cells having rounded shape and area equal to or below 20% of the area of Fc control-treated cells were considered as retracting.

RESULTS

Chemical Library Screening to Identify Compounds That Inhibit Ligand Binding to the EphA4 Receptor—To identify small molecule inhibitors of ligand binding to the EphA4 receptor, we designed an assay that takes advantage of a peptide ligand previously identified by phage display (14). The peptide, designated KYL, has some sequence similarity with the ephrin-A G-H loop, which mediates high affinity binding to Eph receptors (43). Furthermore, the KYL peptide was shown to competitively inhibit ephrin binding to EphA4, suggesting that it targets the high affinity ligand-binding site of the receptor (14). We considered the peptide more suitable for high throughput screening assays than an ephrin-A Fc ligand because it is less expensive to produce and binds to EphA4 with lower affinity, which should facilitate identification of initial hits.

The biotinylated KYL peptide was immobilized on streptavidin-coated ELISA wells, and binding of the extracellular domain of EphA4 fused to alkaline phosphatase (EphA4 AP) was measured in the presence of chemical compounds. We screened 10,000 compounds from the DIVERSet™ library (ChemBridge, Inc.) at 10 μg/ml in a 96-well format, and identified 43 compounds that reproducibly inhibited EphA4 AP binding by more than 50% in both the original screen and a rescreen of the hits (Fig. 1*A*). Four of the compounds shared a 2,5-dimethylpyrrolyl benzene scaffold and inhibited EphA4 AP binding to the KYL peptide with IC_{50} values ranging from 3 to 56 μM (Fig. 1*B*). Importantly, compound **1**, 2-hydroxy-4-(2,5-dimethyl-1-pyrrolyl)benzoic acid, also inhibited binding of ephrin-A5 AP to the EphA4 extracellular domain with an IC_{50} value of 13 μM (Fig. 2). Control experiments also verified that the compound binds reversibly to EphA4 and does not inhibit alkaline phosphatase activity or protein-protein interac-

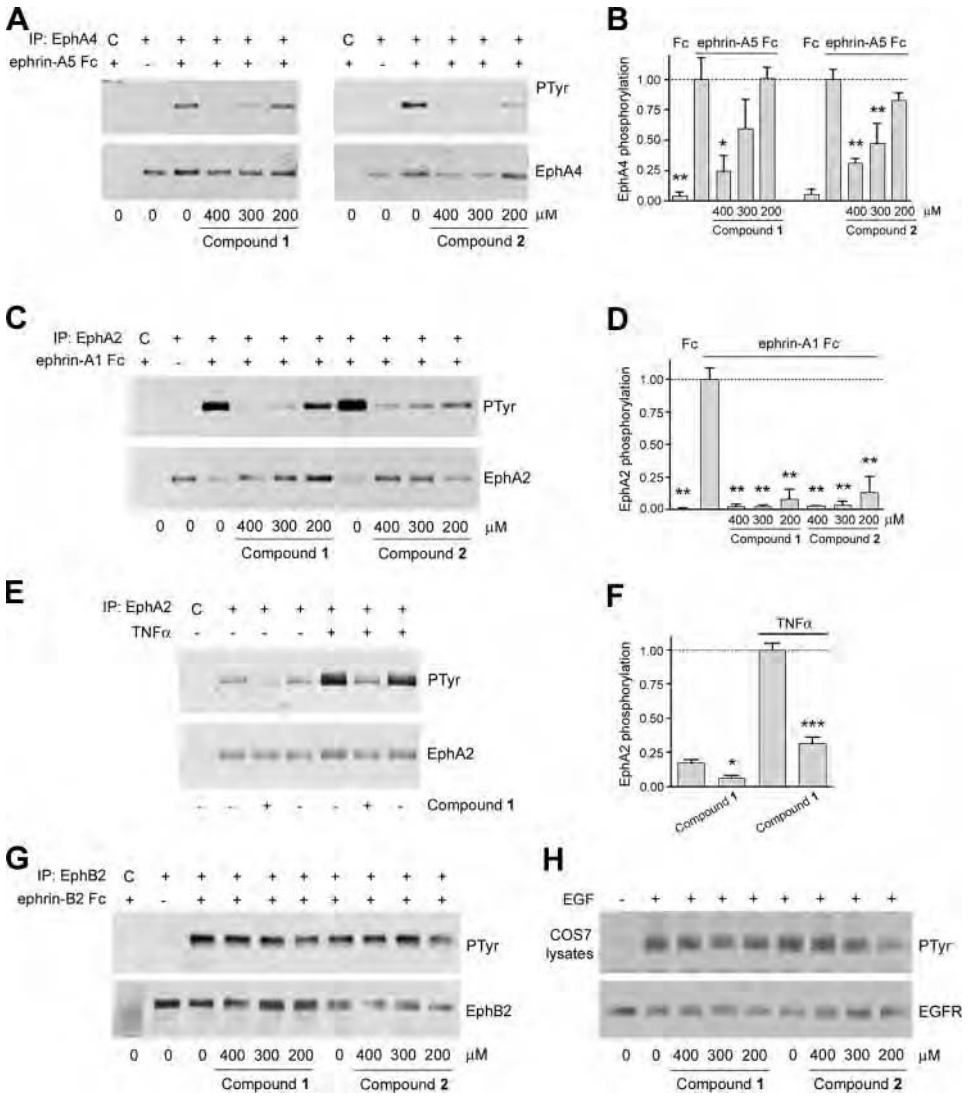


FIGURE 5. **Compounds 1 and 2 inhibit ephrin-induced tyrosine phosphorylation of EphA4 and EphA2.** *A,* HT22 neuronal cells pretreated with the indicated concentrations of compounds **1** or **2** for 15 min were stimulated with 0.5 μg/ml ephrin-A5 Fc (+) or Fc as a control (−) for 20 min in the continued presence of the compounds. EphA4 immunoprecipitates were probed with anti-phosphotyrosine antibody (*PTyr*) and reprobed for EphA4. *C* indicates immunoprecipitations (*IP*) performed with control antibodies from nonimmunized rabbits. *B, histogram* shows the levels of EphA4 phosphorylation quantified from immunoblots and normalized to the amount of immunoprecipitated EphA4. *Error bars* represent standard errors from four experiments for compound **1** and three experiments for compound **2**. Receptor phosphorylation levels were compared with those in ephrin-stimulated cells in the absence of compounds by one-way ANOVA and Dunnett's post test. *, $p < 0.05$; **, $p < 0.01$. *C,* COS7 cells pretreated with the indicated concentrations of compounds **1** or **2** for 15 min were stimulated with 0.1 μg/ml ephrin-A1 Fc or Fc in the continued presence of the compounds. EphA2 immunoprecipitates were probed with anti-phosphotyrosine antibody (*PTyr*) and reprobed for EphA2. *C* indicates control immunoprecipitations. *D, histogram* shows the levels of EphA2 phosphorylation quantified from immunoblots and normalized to the amount of immunoprecipitated EphA2. *Error bars* represent standard errors from two experiments, including some duplicate samples. Statistical analyses were performed as in *B*. *E,* HUVE cells were left unstimulated or stimulated with TNFα for 2 h in the presence of 400 μM compound **1**. Duplicate samples are shown for cells not treated with compound **1**. *C* indicates control immunoprecipitations. EphA2 immunoprecipitates were probed with anti-phosphotyrosine antibody (*PTyr*) and reprobed for EphA2. *F, histogram* shows the levels of EphA2 phosphorylation quantified from immunoblots and normalized to the amount of immunoprecipitated EphA2. *Error bars* represent standard errors from three experiments. Receptor phosphorylation levels in cells treated with compound **1** were compared with those in nontreated samples by nonpaired Student's *t* test. *, $p < 0.05$; ***, $p < 0.001$. *G,* the same protocol described in *C* was used, except that COS7 cells were stimulated with 0.5 μg/ml ephrin-B2 Fc, and the EphB2 receptor was immunoprecipitated. *H,* COS7 cells pretreated with the indicated concentrations of compounds **1** or **2** were stimulated with EGF (+) or left unstimulated (−). Lysates were probed with anti-phosphotyrosine antibody (*PTyr*) and reprobed for the EGF receptor.

tions other than EphA4 ligand binding (data not shown). Thus, compound **1** can inhibit binding of the EphA4 receptor to both a synthetic peptide ligand and a natural ephrin ligand.

Two 2,5-Dimethylpyrrolyl Benzoic Acid Derivatives Selectively Target the EphA4 and EphA2 Receptors—We obtained 49 additional compounds belonging to the same class as compound **1** from ChemBridge and other sources, and we examined them in ELISA experiments for their ability to inhibit EphA4-KYL and EphA4-ephrin-A5 binding. Compound **2,** a 1,2-isomer of compound **1,** also inhibited binding of ephrin-A5 AP to immobilized EphA4 (Fig. 2). The IC_{50} value for inhibition of EphA4-KYL peptide binding by compound **2** was 3 μM and for inhibition of EphA4-ephrin-A5 binding was 9 μM (Fig. 2). By measuring ephrin-A5 AP-binding curves at different compound concentrations, we found that compounds **1** and **2** competitively inhibit EphA4-ephrin-A5 binding with K_i values of 8 and 7 μM, respectively (Fig. 2). These data suggest that compounds **1** and **2** target the high affinity ephrin-binding pocket of EphA4, which is consistent with the chemical shift perturbations caused by compounds **1** and **2** in NMR heteronuclear single quantum coherence spectra of the ephrin-binding domain of EphA4 (see accompanying article, Ref. 74). The K_i value can also be obtained from the IC_{50} value and the dissociation constant (K_D) for receptor-ligand binding, using the Cheng-Prusoff equation (see "Experimental Procedures"). The K_i values for compounds **1** and **2** calculated from the inhibition curves shown in Fig. 2 were 10 and 6 μM, respectively. K_i values calculated from other inhibition curves obtained using different ephrin concentrations ranged from 6 to 10 μM for compound **1** and from 6 to 8 μM for compound **2** (data not shown).

Interestingly, despite the small size of the compounds and the ability of each ephrin ligand to bind promiscuously to different Eph receptors, compounds **1** and **2** preferentially inhibited ephrin binding to EphA4 and EphA2 among the EphA and EphB receptors examined (Fig. 3*A*). Assuming that compound **1** and **2** also competitively inhibit ligand binding to the EphA2 recep-

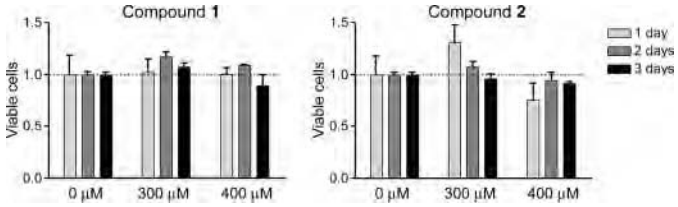


FIGURE 6. **Compounds 1 and 2 do not have toxic effects in cell culture.** HT22 neuronal cells were grown in the presence of the indicated concentrations of compounds **1** and **2** for 1–3 days. Only DMSO was used in the "0 μM" sample, as a control. After addition of MTT, absorbance was measured at 570 nm to determine the levels of viable cells present. The *histograms* show the absorbance obtained for each condition normalized to the absorbance in the absence of the compounds. *Error bars* represent standard errors from triplicate measurements.

tor, the Cheng-Prusoff equation was used to calculate the K_i values for inhibition of EphA2-ephrin-A5 binding, which ranged from 11 to 14 μM for compound **1** and from 10 to 13 μM for compound **2** (data not shown). Both compounds inhibited binding of most ephrin ligands to EphA4, except for ephrin-A4 and ephrin-B2, suggesting differences in how these ephrins bind to EphA4 (we could not examine the effect of the compounds on ephrin-B1 binding because we could not consistently detect binding of this ephrin to EphA4 under the conditions of our assays). Similar selectivity was obtained for EphA2-ephrin-A binding (Fig. 3*B*), suggesting that ephrin-A4 also interacts with EphA2 differently than other ephrins.

Structure-Activity Relationship Analysis of Small Molecules with a 2,5-Dimethylpyrrolyl Benzene Scaffold and Related Compounds—To obtain information that may help improve the potency of compounds **1** and **2**, we measured IC_{50} values for structurally related compounds available from commercial sources or that we synthesized (Fig. 4 and supplemental Fig. 1). Among the 49 analogs initially examined (compounds **5, 6, 8–18,** and **20–55**), none detectably inhibited EphA4-ephrin-A5 binding. Even small changes to the structures of compounds **1** and **2** abolished the ability to inhibit ephrin binding. For example, the presence of the hydroxyl and carboxylic acid moieties and their position on the benzene ring appears to be crucial for the antagonistic activity of the compounds (supplemental Fig. 1). No inhibition of EphA4-ephrin binding was observed with the methyl ester derivative of compound **1** (compound **21**) or when a methoxy group replaced the carboxylic acid group of compound **1** (compound **40**), suggesting that the carboxylic acid group may be involved in hydrogen bonding with EphA4. The two methyl groups on the pyrrole ring also appear to be critical, possibly because they modulate the dihedral angle of the benzene and pyrrole rings or because they contribute to favorable lipophilic interactions with the binding site in EphA4.

Although none of the compounds tested showed measurable inhibition of EphA4-ephrin-A5 binding, their IC_{50} values for inhibition of EphA4-KYL peptide binding were

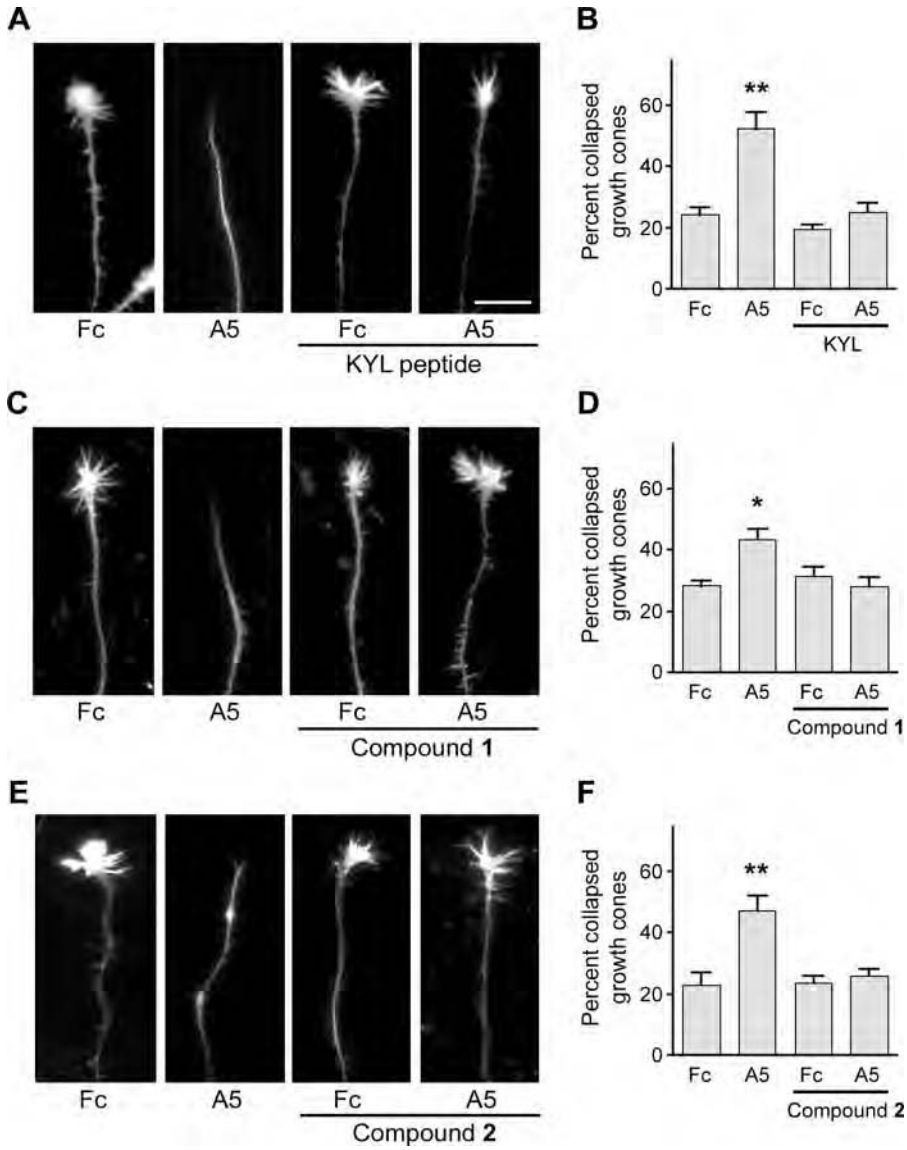
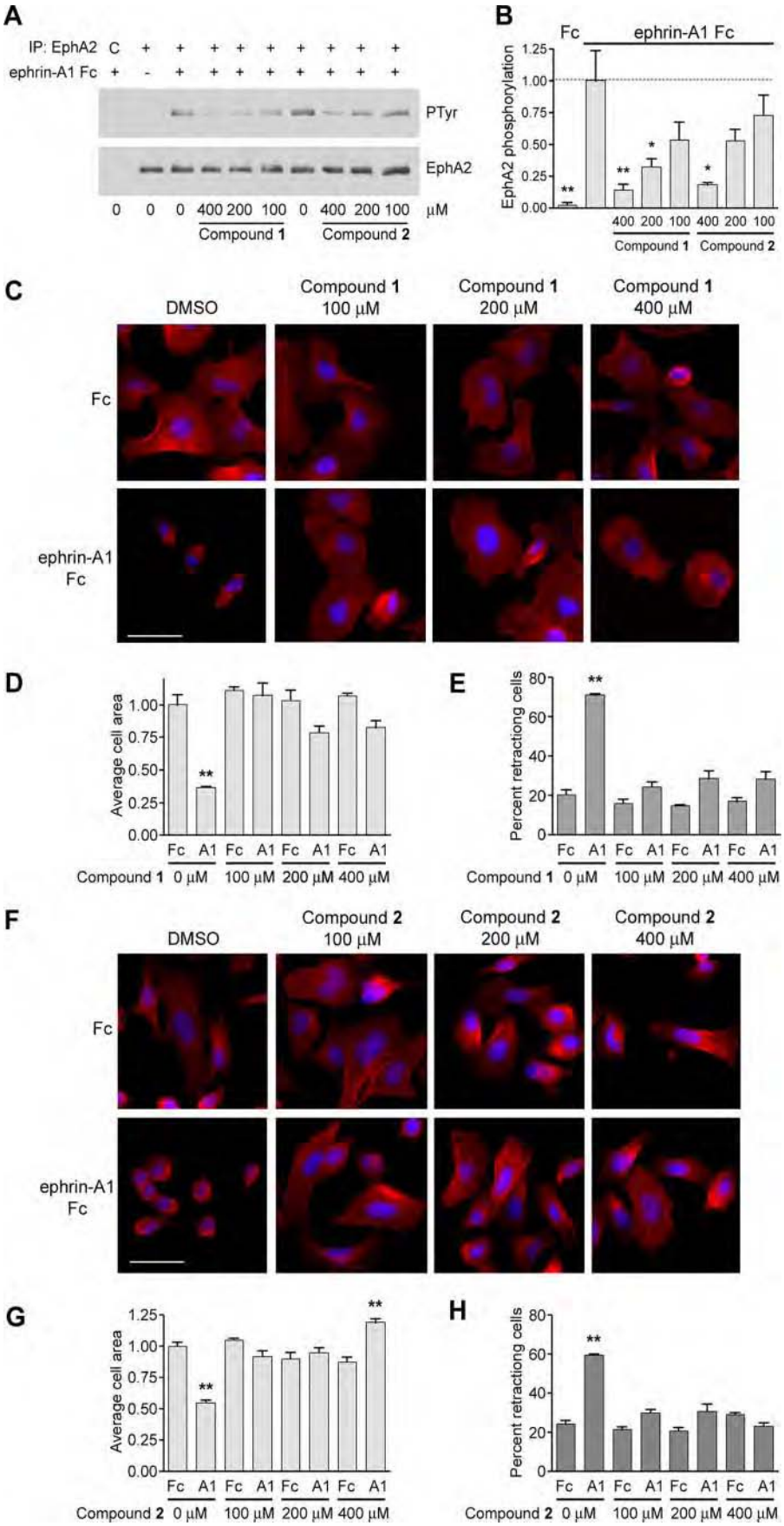


FIGURE 7. **Compounds 1 and 2 block EphA4-dependent growth cone collapse in retinal neurons.** *A,* explants from embryonic day 6 chicken embryonic retina were preincubated with 5 μM KYL peptide for 15 min, stimulated for 30 min with 1 μg/ml ephrin-A5 Fc or Fc as a control in the continued presence of the peptide, and stained with rhodamine-phalloidin to label filamentous actin. *B*, *histogram* showing the mean percentages of collapsed growth cones. Growth cones were scored as collapsed when no lamellipodia or filopodia were visible at the tip of the neurites. In each experiment, 30–200 growth cones per condition were scored, and *error bars* represent standard errors from three experiments. *C–F,* experiments were performed as in *A*, except that retinal explants were treated with 400 μM compound **1** (*C* and *D*) or compound **2** (*E* and *F*). In each experiment, 80–250 growth cones per condition were scored, and *error bars* represent standard errors from three experiments. Collapsed growth cones under different conditions were compared with those in the corresponding Fc control condition by one-way ANOVA and Dunnett's post test. *, $p < 0.05$; **, $p < 0.01$. *Scale bar* in $A = 25$ μm.

used as a guide to design modified versions of compounds **1** and **2** that might have increased potency (Fig. 4). For example, compounds **5**, **6**, and **8**, which have a phenylpropanoic acid, a phenylacetic acid, and phenoxyacetic acid in place of the benzoic acid in compound **25**, inhibited EphA4-KYL binding with 10–40-fold lower IC_{50} values than compound **25**. This suggested that substituting the carboxylic acid group of compound **1** with these other groups might improve its inhibitory activity. We therefore synthesized compounds **3** and **4**. The phenolic hydroxyl group highly improved the ability of compounds **3** and **4** to inhibit ephrin-A5 binding compared with compounds **6** and **5**, which lack the hydroxyl and did not show any activity against ephrin-A5. Compounds **3** and **4** are still selective EphA4 and EphA2 inhibitors and show the same differential inhibition of ephrin binding as compounds **1** and **2** (data not shown). However, inhibition of EphA4-KYL binding was not greatly affected by the phenolic hydroxyl group, suggesting that this group is more important for inhibition of ephrin-A5 binding than peptide binding. This was confirmed by the lack of activity with ephrin-A5 of compound **7**, a methyl ether of compound **4**, which, however, inhibited EphA4-KYL binding with a relatively low IC_{50} value. It is also interesting that despite being able to inhibit ephrin-A5 binding to EphA4, compound **3** inhibited EphA4-KYL binding less effectively than compounds **5**–**7**, which do not measurably inhibit ephrin binding. This suggests that somewhat different structural features may be required for inhibition of EphA4 interaction with ephrin-A5 *versus* the KYL peptide.

The IC_{50} values for compounds **10** and **15** were ~6- and 3-fold lower than those for compounds **25** and **30**, which only differ for the absence of a methyl group attached to the benzene ring. This suggested that adding a methyl group to the benzene ring of compounds **1** and **2**



may improve their inhibitory activity. We therefore synthesized compound **19**, which corresponds to compound **2**, with an additional methyl group as a substituent on the benzene ring. However, compound **19** did not inhibit EphA4-ephrin-A5 binding and inhibited EphA4-KYL binding only when present at high concentration. Perhaps the ability of the methyl group to enhance the activity of compounds **10** and **15** depends on its position with respect to the other substituents on the benzene ring. If this is true, the synthesis of alternative compounds carrying the methyl group at different positions may give different results.

Compounds **1** *and* **2** *Selectively Inhibit EphA4 and EphA2 Activation by Ephrin in Cells without Showing Toxicity*—Compounds **1** and **2** were the best antagonists in the ELISAs. Therefore, we examined the ability of these two compounds to inhibit ephrin-induced EphA4 and EphA2 tyrosine phosphorylation (indicative of receptor activation) in cultured cells. Both compounds blocked tyrosine phosphorylation of endogenous EphA4 in HT22 neuronal cells stimulated with ephrin-A5 Fc, although the concentrations needed were higher than those effective in the ELISAs (Fig. 5, *A* and *B*). The compounds also inhibited tyrosine phosphorylation of endogenous EphA2 in COS7 cells stimulated with ephrin-A1 Fc (Fig. 5, *C* and *D*) and in HUVE cells treated with TNFα to stimulate expression of endogenous ephrin-A1 (Fig. 5, *E* and *F*) (44–46). The TNFα-dependent increase in EphA2 phosphorylation did not occur in cells treated with the protein synthesis inhibitor cycloheximide, consistent with a TNFα-mediated up-regulation of ephrin-A1 expression (data not shown). Furthermore, the compounds prevented ephrin-dependent degradation of EphA2 (47), as expected from inhibition of ephrin binding. Consistent with the selectivity observed in the ELISAs, compounds **1** and **2** did not inhibit EphA4 phosphorylation in cells stimulated with ephrin-A4 Fc (data not shown) or phosphorylation of endogenous EphB2 in COS7 cells stimulated with ephrin-B2 Fc (Fig. 5*G*). Moreover, the compounds did not inhibit phosphorylation of the EGF receptor in COS7 cells stimulated with EGF (Fig. 5*H*) or overall tyrosine phosphorylation in COS and HT22 cells (data not shown). Assessment of cell viability using the MTT assay did not reveal any toxicity of compounds **1** and **2** at concentrations up to 400 μM for several days (Fig. 6).

Compounds **1** *and* **2** *Inhibit EphA4-dependent Growth Cone Collapse in Retinal Neurons*—Growth cones are enlarged structures at the leading edge of axons and control the growth of the axons toward their synaptic targets by responding to environmental cues (48, 49). The growth cones of chicken retinal neurites are well known to collapse in response to ephrin-A ligand stimulation (50, 51). Because EphA4 is homogeneously expressed in different parts of the retina, whereas other EphA receptors are preferentially expressed in the temporal but not the nasal region of the retina (52), EphA4 is the predominant EphA receptor in nasal retinal neurons. Therefore, we used explants from the chicken nasal retinal to examine the ability of compounds **1** and **2** to counteract EphA4-mediated growth cone collapse. Although co-expression of ephrin-A ligands with EphA4 in the nasal retina makes the growth cones less sensitive to the collapsing effects of ephrin-A5 Fc, the growth cones still collapse when exposed to high concentrations of the ephrin (50–52). The KYL peptide, which has been shown to selectively inhibit EphA4-ephrin binding (14), blocked collapse of nasal growth cones stimulated with ephrin-A5, confirming the requirement for EphA4 activation (Fig. 6, *A* and *B*). Compound **1** (Fig. 7, *C* and *D*) and compound **2** (Fig. 7, *E* and *F*) also blocked the growth cone collapsing effects of ephrin-A5 Fc. Importantly, despite the sensitivity of growth cones to their surrounding environment (48, 49), neither the KYL peptide nor the two compounds at concentrations as high as 400 μM affected the shape of unstimulated growth cones.

Compounds **1** *and* **2** *Inhibit EphA2-dependent Retraction of the Cell Periphery*—EphA2 is known to induce changes in cell morphology when activated by ephrin-A1, including retraction of the cell periphery and cell rounding (53, 54). Because EphA2 is the predominant EphA receptor expressed in PC3 prostate cancer cells (55), we used these cells to examine whether compounds **1** and **2** are able to inhibit EphA2-mediated cell retraction. Treatment with the compounds blocked EphA2 activation following stimulation with ephrin-A1 Fc (Fig. 8, *A* and *B*) as well as the decrease in cell spreading (Fig. 8, *C, D, F,* and *G*) and the increase in the percentage of rounded cells (Fig. 8, *C, E, F,* and *H*) caused by ephrin-A1 Fc stimulation. Importantly, the compounds did not affect cell morphology in the absence of ephrin treatment (Fig. 8, *C–H*).

DISCUSSION

We report here for the first time the identification of small molecules that inhibit the interaction between Eph receptors and ephrins. To isolate small molecule inhibitors of EphA4, we performed a high throughput screening designed to identify compounds that inhibit ligand binding to this receptor. These inhibitors are advantageous compared with standard tyrosine kinase inhibitors because they can act without penetrating inside the cell and can be more selective. Among the many Eph receptors tested, the two 2,5-dimethylpyrrolyl benzoic acid derivatives that we have identified indeed show preferential

FIGURE 8. **Compounds 1 and 2 inhibit EphA2-dependent retraction and rounding of PC3 prostate cancer cells.** *A,* PC3 cells pretreated for 15 min with the indicated concentrations of compounds **1** or **2** were stimulated with 0.5 μg/ml ephrin-A1 Fc (+) or Fc as a control (−) for 20 min in the continued presence of the compounds. EphA2 immunoprecipitates (*IP*) were probed with anti-phosphotyrosine antibody (*PTyr*) and reprobed for EphA2. *C* indicates immunoprecipitations performed with control antibodies. *B, histogram* showing the levels of EphA2 phosphorylation normalized to the amount of immunoprecipitated EphA2. *Error bars* represent standard errors from three experiments. Receptor phosphorylation levels were compared with those in the ephrin-stimulated cells in the absence of compounds by one-way ANOVA and Dunnett's post test. *C,* PC3 cells stimulated with compound **1** as in *A* were stained with rhodamine-phalloidin to label actin filaments (*red*) and 4′,6-diamidino-2-phenylindole (*DAPI*) to label nuclei (*blue*). DMSO was used as a control (0 μM). *D, histogram* showing the average area of the cells normalized to the value obtained for the Fc-treated cells. *E, histogram* showing the average percentage of retracting cells. Cells having rounded shape and area less than 20% of the mean value obtained for the Fc-stimulated cells were scored as retracting. *Error bars* in *D* and *E* represent standard errors from three experiments. *F–H,* same experiments and analyses as in *C–E* were performed using compound **2**. The areas occupied by the cells and the percentage of cell retraction under different conditions were compared with those in the Fc control condition by one-way ANOVA and Dunnett's post test. *, $p < 0.05$; **, $p < 0.01$. *Scale bars* in *C* and *F* = 50 μm.

inhibition of only two Eph receptors, EphA4 and the closely related EphA2. Our results also suggest that the two compounds are competitive inhibitors that target the high affinity ligand binding pocket of the receptors, a conclusion that is supported by NMR studies with EphA4 (74).

Given the small size of the two dimethylpyrrole derivatives compared with the ephrin binding pocket, their selectivity for EphA4 and EphA2 is particularly interesting and suggests that these compounds target a region that is not highly conserved in other Eph receptors. The two dimethylpyrrole derivatives also show selectivity with regard to ephrin binding, because they inhibited association of most ephrins tested except for ephrin-A4 and ephrin-B2, even when used at high concentrations. This suggests that these ephrins bind differently to the receptors compared with other ephrins of the same class. For example, interfaces not involving the ephrin-binding pocket may be of higher affinity with ephrin-A4 and ephrin-B2 than with other ephrins. Alternatively, there may be differences in the binding of ephrin-A4 and ephrin-B2 to the ephrin-binding pocket despite the similarity of the G-H loops of these ephrins with those of other ephrins whose binding is inhibited by the compounds. Structural studies will be necessary to elucidate how different ephrins interact with EphA4 and EphA2. The selectivity of the two dimethylpyrrole derivatives toward different Eph receptors and ephrins was confirmed in cell-based assays, where the addition of the compounds selectively blocked the ephrin-dependent tyrosine phosphorylation of EphA4 and EphA2 but not EphB2. The compounds also had no effect on the EGF-dependent phosphorylation of the EGF receptor, which is instead inhibited by many of the small molecules targeting kinase domains (19) and by epigallocatechin gallate (56).

The two pyrrole derivatives, like the KYL peptide, also blocked EphA4-mediated growth cone collapse in retina explants, suggesting that the compounds and the KYL peptide could promote axon growth. Interestingly, EphA4 has been proposed to play multiple roles in the inhibition of spinal cord regeneration after injury. In mouse and rat models of spinal cord injury, expression of this receptor is up-regulated in both glial cells and neurons near the site of injury (27, 28). EphA4 expressed in the reactive glial cells may act as a negative regulator of axon regeneration by favoring the formation of the glial scar and by stimulating ephrin-B reverse signaling in axons. Furthermore, EphA4 expressed in the damaged axons may interact with ephrin-B2 expressed in the surrounding astrocytes and ephrin-B3 expressed in myelin, leading to inhibition of axon sprouting and outgrowth (28, 57). The relative importance of these different effects is not yet known; however, some data suggest that inhibiting EphA4 function may be beneficial for the treatment of spinal cord injuries. For example, it has been reported that EphA4 knock-out mice have a significantly reduced glial scar and improved ability to regenerate spinal cord connections after spinal cord injury (27). In addition, a recent study has shown that the KYL peptide protects rat neocortical growth cones from collapsing after ephrin-A5 Fc treatment and that infusion of the peptide (14) into the lesioned spinal cord enhances axon sprouting, reduces cavity formation, and improves behavioral recovery (29). Inhibition of retinal growth cone collapse by the two dimethylpyrrole derivatives is an encouraging result that suggests that similar compounds with higher affinity could be used to enhance axon regrowth after injury. Inhibition of EphA4-ephrin interaction could also be useful in neuropathologies characterized by dendritic spine loss in the brain (14, 58), to promote blood clotting (30), and to inhibit some forms of cancer (32, 33, 35).

The other Eph receptor preferentially targeted by the two dimethylpyrrole derivatives, EphA2, is widely expressed in many types of cancer cells and in the tumor vasculature (15, 59, 60). The dimethylpyrrole derivatives inhibit EphA2-dependent retraction and rounding of prostate cancer cells stimulated with exogenous ephrin-A1 Fc, suggesting that treatment with the compounds can inhibit the functional effects of EphA2. Interestingly, the compounds completely reverted the effect of ephrin-A1 treatment on cell retraction and rounding at concentrations that only partially inhibited EphA2 tyrosine phosphorylation, suggesting that high levels of EphA2 activation may be required to promote changes in cell adhesion and morphology. Inhibiting EphA2-ephrin binding in cancer cells is expected to be useful in the cases where EphA2 is highly activated and its signaling activity promotes tumorigenesis (61–63), but not in other cases where the tumor cells express low levels of endogenous ephrin-A1 (64). However, the most exciting application of EphA2-targeting molecules is for inhibition of tumor angiogenesis and other forms of pathological angiogenesis (65–71). Importantly, EphA2 is expressed in adult angiogenic blood vessels, but not in embryonic or adult quiescent blood vessels (60, 72), consistent with evidence that targeting the pathological effects of EphA2 does not affect the normal vasculature. Unlike the previously identified EphA2-targeting peptides, which inhibit EphA2-ephrin binding in ELISAs but stimulate EphA2 phosphorylation in cells (13), the dimethylpyrrole derivatives also inhibit EphA2 activation in cells, including endothelial cells treated with the angiogenic factor TNFα (45). Thus, this class of compounds may be further developed for inhibition of pathological forms of angiogenesis, similar to the EphA receptor Fc fusion proteins that have been successfully used to inhibit angiogenesis in mouse tumor models and in a rat model of retinopathy of prematurity (65, 66, 70, 73).

Analysis of the structure-activity relationship of many analogs of the dimethylpyrrole derivatives for inhibition of EphA4-KYL peptide binding did not lead to the identification of compounds inhibiting EphA4-ephrin interaction with improved potency. However, the rational design of other analogs with improved potency should now be possible based on the three-dimensional structure of EphA4 in complex with compounds **1** and **2**, which provides valuable insight into the molecular interactions of the compounds with the receptor (74).

Pharmacological tools to manipulate Eph receptor function will open new avenues of research and therapy. The compounds identified in this study may be used as leads to develop pharmaceuticals for the treatment of pathologies caused by dysregulation of EphA2 and EphA4 function. Importantly, our results provide evidence that the high affinity Eph receptor-ephrin interface can be successfully targeted by inhibitory small molecules and demonstrate the feasibility of approaches to

identify ligand-binding inhibitors for the Eph receptors, which may also have wide application with other families of receptors.

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Cell

Leading Edge
Review

Eph-Ephrin Bidirectional Signaling in Physiology and Disease

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Receptor tyrosine kinases of the Eph family bind to cell surface-associated ephrin ligands on neighboring cells. The ensuing bidirectional signals have emerged as a major form of contact-dependent communication between cells. New findings reveal that Eph receptors and ephrins coordinate not only developmental processes but also the normal physiology and homeostasis of many adult organs. Imbalance of Eph/ephrin function may therefore contribute to a variety of diseases. The challenge now is to better understand the complex and seemingly paradoxical signaling mechanisms of Eph receptors and ephrins, which will enable effective strategies to target these proteins in the treatment of diseases such as diabetes and cancer.

Eph-Ephrin Bidirectional Signaling

Since its discovery two decades ago, the Eph family of receptor tyrosine kinases has been implicated in an increasing number of physiological and pathological processes in many cell types and different organs. Therefore, elucidating the mechanism of action of the Eph receptors and their signaling networks is important for understanding developmental processes, the physiology of adult organs and, as is becoming increasingly evident, the pathogenesis of many diseases. Eph receptors have diverse activities, including widespread effects on the actin cytoskeleton, cell-substrate adhesion, intercellular junctions, cell shape, and cell movement (Egea and Klein, 2007; Himanen et al., 2007; Pasquale, 2005). In addition, effects on cell proliferation, survival, differentiation, and secretion have also been described. These activities depend on the interaction of the Eph receptors with the ephrins (*Eph* r*eceptor in*teracting proteins). In the human genome, there are nine EphA receptors that bind to five GPI-linked ephrin-A ligands and five EphB receptors that bind to three transmembrane ephrin-B ligands. Interactions are promiscuous within each class, and some Eph receptors can also bind to ephrins of the other class.

Several of the domains in the Eph receptor extracellular region can bind to the ephrins. The amino-terminal "ephrin-binding" domain contains a high-affinity binding site that mediates receptor-ephrin interaction between cells (Figure 1) (Himanen et al., 2007; Wimmer-Kleikamp and Lackmann, 2005). Two additional lower-affinity ephrin-binding sites have also been identified in the ephrin-binding domain and the cysteine-rich region, which are thought to facilitate clustering of multiple Eph-ephrin complexes. The Eph fibronectin type III domain closer to the membrane can also bind to ephrins, if they are located on the same cell surface.

Downstream Signaling

A distinctive feature of Eph-ephrin complexes is their ability to generate bidirectional signals that affect both the receptor-expressing and ephrin-expressing cells (Pasquale, 2005). Eph receptor "forward" signaling depends on the tyrosine kinase domain, which mediates autophosphorylation as well as phosphorylation of other proteins, and on the associations of the receptor with various effector proteins. Ephrin-B "reverse" signaling also depends in part on tyrosine phosphorylation of the ephrin cytoplasmic region (mediated by Src family kinases and some receptor tyrosine kinases) and on associated proteins. Most Eph receptors and the B-type ephrins also have a carboxy-terminal PDZ domain-binding site, which is particularly important for the physiological functions of ephrin-B (Egea and Klein, 2007). The mechanisms of reverse signaling for ephrin-A are less understood, but these GPI-linked ephrins probably use associated transmembrane proteins to fulfill their signaling function. Several candidates have been reported at meetings, including the p75 low-affinity nerve growth factor receptor (T.R. McLaughlin et al., 2007, Soc. Neurosci., abstract).

Eph receptors and ephrins use some common signaling effectors, such as Src family kinases and Ras/Rho family GTPases, which are particularly important for the organization of the actin cytoskeleton and cell adhesion (Figure 1). Some signaling connections may apply only to a particular Eph class, including those between EphA receptors and the Rho exchange factor Ephexin or between EphB receptors and the exchange factors Intersectin and Kalirin. Others are more selective. For example, the lipid phosphatase Ship2 was found to interact only with EphA2, and the GTPase-activating proteins SPAR/E6TP1 interacted only with EphA4 and EphA6 among several EphA and EphB receptors examined (Richter et al., 2007; Zhuang et al., 2007).

An emerging theme is that Eph receptors and ephrins activate complex bidirectional signaling networks that often include signaling pathways with opposite effects (Figure 1). This may explain why differences in cellular context can dramatically alter the outcome of Eph/ephrin stimulation. Furthermore, the degree of Eph/ephrin clustering may not only affect signal strength but may also differentially regulate

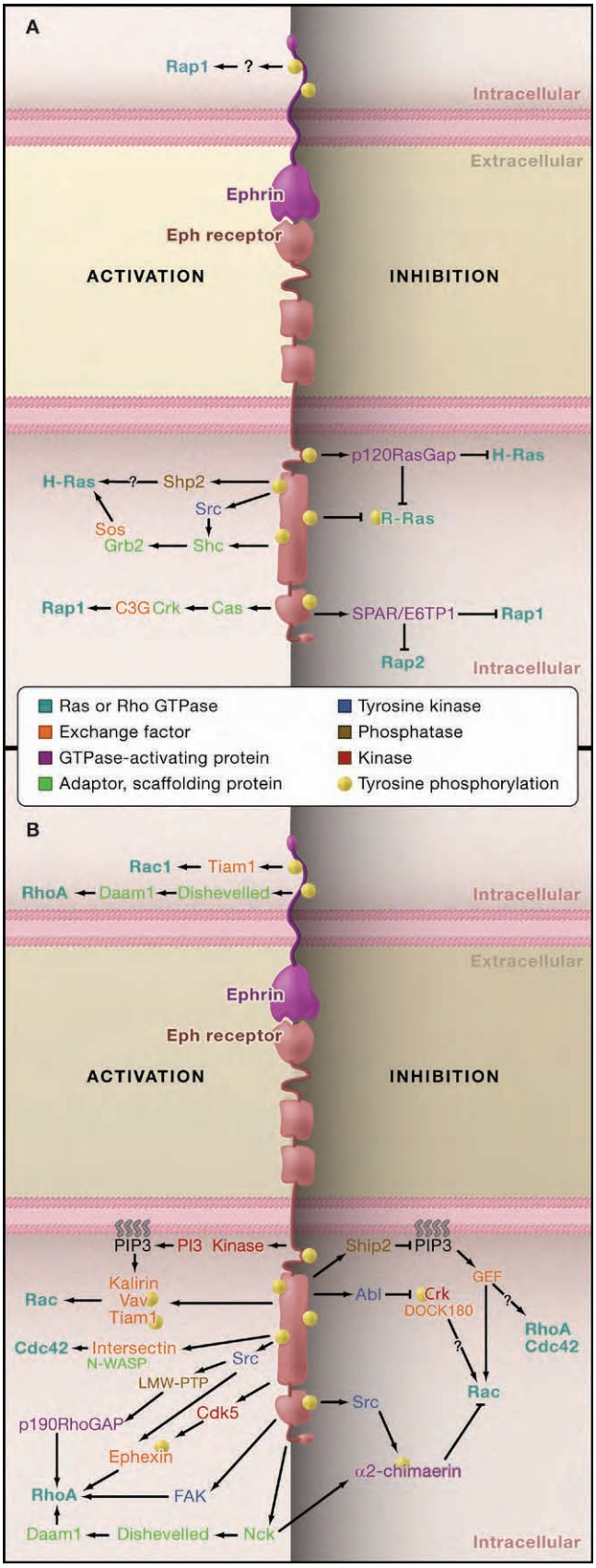

Figure 1. Eph Receptor-Ephrin Bidirectional Pathways Regulate GTPases

(A) Regulation of Ras GTPases. (B) Regulation of Rho GTPases. The domain structure of an Eph receptor is shown schematically, including from the N terminus: ephrin-binding domain, cystein-rich region, two fibronectin type III domains, transmembrane segment, juxtamembrane domain, kinase domain, SAM domain, and PDZ domain-binding site. The domain structure of an ephrin-B ligand is also shown, including the Eph-binding domain, linker region, transmembrane segment, cytoplasmic region, and PDZ domain-binding site. The pathways shown have been characterized with one or several Eph receptors/ephrins. For example, in (A) Shp2 has been linked to EphA2; Shc-Grb2 to EphA2 and EphB1; Cas-Rap1 to EphB1; and SPAR/E6TP1 to EphA4 and EphA6. In (B), α2-chimaerin has been linked to EphA4; FAK to EphA2 and EphB2; Ship2 to EphA2; Abl-Crk to EphB4; Ephexin family members to EphA receptors; and Kalirin, Tiam1, and Intersectin to EphB receptors. Tyrosine phosphorylation is shown only for some effectors where it has a demonstrated role in Eph-ephrin bidirectional signaling. The location of the arrows does not imply the involvement of a particular Eph or ephrin domain. The relative activation of different pathways and their effects on cell behavior may depend on the ephrin levels, degree of receptor clustering, and cellular context. The question marks indicate signaling connections that have not been conclusively demonstrated downstream of Eph receptors or ephrins. PIP3, phosphatidylinositol (3,4,5) phosphate; GEF, guanine nucleotide exchange factor; LMW-PTP, low-molecular-weight phosphotyrosine phosphatase.

downstream pathways thus leading to variable outcomes (Pasquale, 2005; Poliakov et al., 2004). Further increasing versatility, forward and reverse signaling can also be independently regulated, for example through Eph receptor dephosphorylation (Konstantinova et al., 2007). In addition, interactions between Eph receptors and ephrins located on the same cell surface appear to represent a mechanism for silencing bidirectional signaling, although it is unclear under what circumstances Eph receptors and ephrins intermingle rather than segregate in different microdomains of the plasma membrane (Egea and Klein, 2007).

Processing of Eph-Ephrin Complexes

A well-characterized effect of Eph forward signaling is retraction of the cell periphery following contact with ephrin-expressing cells (Pasquale, 2005). This repulsive response is particularly important for axon guidance and sorting of Eph-expressing cells from ephrin-expressing cells during development. Several mechanisms can explain how the initial adhesive contact evolves into cell separation. One is removal of the adhesive Eph-ephrin complexes from the cell surface by endocytosis of vesicles containing plasma membrane fragments derived from both cells (Egea and Klein, 2007). An implication of this unusual mechanism is that the two cells exchange Eph receptors or ephrins and possibly their associated proteins, which may continue to signal from intracellular compartments. Another way to convert cell adhesion into repulsion is proteolytic cleavage (Egea and Klein, 2007; Himanen et al., 2007). Studies have shown that metalloproteases and other proteases can cleave the extracellular portions of EphB receptors and ephrins. The remaining membrane-anchored fragments are further cleaved by γ-secretase, followed by proteasomal degradation.

Proteolytic cleavage not only terminates the adhesive Eph-ephrin interaction and causes downregulation of the proteins, but it can also generate Eph/ephrin fragments with new activities. For example, the ephrin-B cytoplasmic pep-

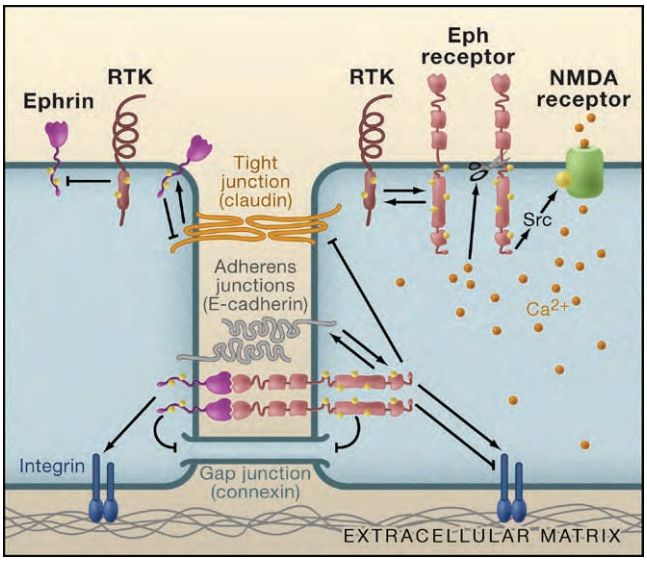

Figure 2. Crosstalk between Eph-Ephrins and Other Receptors
Some forms of crosstalk occur at epithelial cell junctions, others have been reported in neurons and other cell types. RTK, receptor tyrosine kinase; yellow circles, tyrosine phosphorylation; the scissors indicate proteolytic cleavage.

tide released by γ-secretase activates the tyrosine kinase Src, which in turn phosphorylates the cytoplasmic domain of intact B-type ephrins and perhaps other substrates (Egea and Klein, 2007). Furthermore, the soluble Eph and ephrin extracellular portions released by metalloproteases might reach distant cells and trigger effects that are independent of cell-cell contact. They could, for example, function as monomeric inhibitors of bidirectional signaling. Alternatively, soluble A-type ephrins oligomerized by transglutamination may serve to activate EphA receptors at a distance (Alford et al., 2007).

Crosstalk with Other Signaling Systems

Although bidirectional signaling is their best characterized modus operandi, Eph receptor and ephrins may also function independently of each other and/or in concert with other cell-surface communication systems (Figure 2). For example, recent studies have proposed that members of the epidermal growth factor (EGF) receptor family can coopt EphA2 as an effector to promote cell motility and proliferation, perhaps independently of ephrin stimulation (Brantley-Sieders et al., 2008; Larsen et al., 2007). Other studies have shown association and synergistic responses of fibroblast growth factor (FGF) receptors and EphA4, and that phosphorylation by FGF receptors inhibits ephrin-B1 activities (Arvanitis and Davy, 2008). Intricate links between EphB/ephrin-B and Wnt signaling have also been revealed in different model systems. EphB receptors and Ryk, a Wnt receptor containing an inactive tyrosine kinase domain, can physically associate and likely function together in craniofacial development and axon guidance (Arvanitis and Davy, 2008; Schmitt et al., 2006). Furthermore, both EphB receptors and B-type ephrins can signal through components of the noncanonical Wnt pathway (Figure 1B) (Kida et al., 2007; Lee et al., 2006). This pathway in turn causes endocytic removal of EphB receptors from the cell surface, whereas canonical Wnt signaling upregulates EphB transcripts and downregulates ephrin-B transcripts (Clevers and Batlle, 2006; Kida et al., 2007).

E-cadherin-dependent intercellular adhesion can also regulate Eph receptor expression, cell-surface localization, and ephrin-dependent activation (Arvanitis and Davy, 2008; Ireton and Chen, 2005). The regulation is reciprocal, and EphB signaling drives E-cadherin to the cell surface thus promoting the formation of epithelial adherens junctions and enabling EphB/ephrin-B-dependent cell sorting. Conversely, inhibiting EphB-ephrin-B binding was found to disturb adherens junctions (Cortina et al., 2007; Noren and Pasquale, 2007). EphA2 overexpression, on the other hand, has been shown to destabilize adherens junctions through a pathway involving Src, the low-molecular-weight phosphotyrosine phosphatase, and p190RhoGAP, resulting in increased RhoA activity (Figure 1B) (Fang et al., 2008). The Eph system also affects integrin-mediated cell communication with the extracellular environment (Bourgin et al., 2007; Pasquale, 2005; Wimmer-Kleikamp and Lackmann, 2005).

Crosstalk of EphA2 or ephrin-B1 with claudins, which are components of epithelial tight junctions, has been implicated in the regulation of cell adhesion and intercellular permeability (Arvanitis and Davy, 2008). Some claudins can also cause ephrin-B1 tyrosine phosphorylation independently of EphB receptors. Gap junction proteins are also critical for Eph/ephrin function in cell sorting, insulin secretion, and osteogenic differentiation (Davy et al., 2006; Konstantinova et al., 2007; Poliakov et al., 2004).

Reciprocal communication also occurs between EphB receptors and calcium channels (Figure 2). Following ephrin binding, EphB2 associates with the NMDA receptors, which are calcium channels, and promotes clustering of these neurotransmitter receptors at synapses (Yamaguchi and Pasquale, 2004). Moreover, activation of Src family kinases downstream of EphB2 leads to NMDA receptor phosphorylation, which increases NMDA-dependent calcium influx. Interestingly, increased intracellular calcium in turn promotes proteolytic degradation of EphB2, demonstrating that Eph levels can be regulated by intracellular calcium independently of ephrin binding (Litterst et al., 2007).

More information on Eph signaling mechanisms and crosstalk with other signaling systems can be found in recent reviews (Arvanitis and Davy, 2008; Egea and Klein, 2007; Himanen et al., 2007; Noren and Pasquale, 2004; Pasquale, 2005; Poliakov et al., 2004).

Neural Development, Plasticity, and Regeneration

The activities of Eph receptors and ephrins in the nervous system have been extensively studied. Neurons form complex networks where electrical signals travel from axonal to dendritic processes through specialized junctions called synapses. Here, neurotransmitters released from the presynaptic terminal in response to electrical signals activate postsynaptic ion channel receptors that initiate new electrical and chemical signals in the postsynaptic neuron. The network of neuronal processes is embedded among surrounding glial cells, which regulate many properties of the neurons including their

ability to form synapses. Eph-ephrin bidirectional signaling is important not only for the communication between neurons but also for that between neurons and glial cells (Yamaguchi and Pasquale, 2004).

Development of Neuronal Connections

Eph receptors and ephrins are highly expressed in the developing nervous system, where they have well-known roles in the establishment of neuronal connectivity by guiding axons to the appropriate targets and regulating the formation of synaptic connections. The trajectories of many axonal projections depend on Eph receptors and ephrins distributed in gradients or forming boundaries (Luo and Flanagan, 2007; Pasquale, 2005; Poliakov et al., 2004). A number of Ras/Rho regulatory proteins have been implicated over the years in axon guidance by the Eph receptors, including several guanine nucleotide exchange factors for Rho GTPases (Figure 1B). Only recently four simultaneous studies have also implicated a GTPase-activating protein for Rac1, α2-chimaerin, as a critical EphA4 effector (Beg et al., 2007; Iwasato et al., 2007; Shi et al., 2007; Wegmeyer et al., 2007). Remarkably, α2-chimaerin mutant mice have defects in the formation of cortical and spinal motor circuits that phenocopy those in the EphA4 knockout mice, indicating that α2-chimaerin is essential for certain axon guidance decisions that depend on EphA4. Mice lacking the adaptor proteins Nck1 and Nck2 in the nervous system also exhibited similar defects, suggesting that Nck adaptors, which can bind both EphA4 and α2-chimaerin, may also play a role in the pathway (Fawcett et al., 2007; Wegmeyer et al., 2007).

In vitro and in vivo analyses of hippocampal and cortical neurons have revealed that the EphB receptors and B-type ephrins regulate multiple steps in the assembly and maturation of the pre- and postsynaptic sides of excitatory synapses. Interestingly, different Eph receptor domains can control different aspects of synaptogenesis. The EphB2 extracellular region, for example, is sufficient to promote the assembly of presynaptic structures even when expressed in non-neuronal cells (Kayser et al., 2006). This activity requires the ephrin-binding domain, suggesting a *trans*-synaptic interaction with axonal ephrins. This ability of EphB2 to promote presynaptic specializations, however, may vary in different brain regions because it was detected in cortical but not hippocampal neurons. Activation of ephrin-B reverse signaling by postsynaptic EphB2 has also been recently implicated in the morphological and functional maturation of developing retinotectal synapses in the *Xenopus* optic tectum (Lim et al., 2008). The EphB2 extracellular portion also associates with NMDA neurotransmitter receptors and promotes their clustering at synapses following ephrin-B stimulation (Dalva et al., 2007). Furthermore, EphB2 promotes AMPA neurotransmitter receptor clustering and endocytosis, and these activities respectively depend on the PDZ domain-binding site of EphB2 and its kinase activity.

Most excitatory synapses are located on small dendritic protrusions called dendritic spines, which compartmentalize the postsynaptic space from the dendritic shaft, but some are also located on the dendritic shaft (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). EphB receptors selectively promote the formation of the synapses located on spines and also play a critical role in spine maturation, which results in the characteristic mushroom shape determined by the actin cytoskeleton. Studies with cultured neurons have implicated several nucleotide exchange factors for Rho GTPases in EphB-dependent spine elaboration, including Kalirin, Intersectin, and Tiam1 (Figure 1B) (Tolias et al., 2007; Yamaguchi and Pasquale, 2004). It is not known whether these exchange factors function in different subsets of dendritic spines and whether there are differences in their effects on the spines.

Ephrin-B ligands are also found postsynaptically, and ephrin-B3 expressed in non-neuronal cells can drive the formation of presynaptic structures in cocultured neurons, presumably by interacting with axonal Eph receptors (Aoto et al., 2007). Interestingly, ephrin-B3 overexpression and knockdown using short-interfering RNAs (siRNAs) in cultured hippocampal neurons have shown that the excitatory synapses induced by ephrin-B3 are located on the dendritic shaft. Consistent with this, the ephrin-B3 knockout mice have fewer shaft synapses in hippocampal area CA1 than wild-type mice. The synaptogenic activity of ephrin-B3 depends on the scaffolding protein GRIP1, which may help ephrin-B3 clustering by interacting with its PDZ domain-binding site. Treatment of cultured hippocampal neurons with EphB2 Fc (a soluble form of the EphB2 extracellular region dimerized by fusion with the Fc portion of an antibody) has also been shown to promote synapse formation and dendritic spine maturation, presumably through ephrin-B1 and/or ephrin-B2 and a reverse signaling mechanism involving recruitment of the adaptors Grb4 and GIT1 (Segura et al., 2007).

It will be interesting to further investigate the involvement of the Eph system in process extension and synaptogenesis of the new neurons that continue to be generated in the hippocampus and the olfactory system throughout life (Chumley et al., 2007). In particular, the integration of newly generated neurons in the hippocampal circuitry seems to be important for the behavioral effects of antidepressants, an area where the involvement of Eph receptors has not yet been explored (Sahay and Hen, 2007).

Plasticity of Neuronal Circuits

Eph receptors and ephrins persist in the adult brain, particularly in regions where neuronal circuits continue to be remodeled in response to environmental changes (Yamaguchi and Pasquale, 2004). Indeed, studies with mutant mice have shown that the Eph system regulates the plasticity of neuronal connections in structures such as the hippocampus, where changes in synapse number and size are important for learning and memory. Although the synaptic localization of Eph receptors and ephrins has not been fully characterized, it is becoming apparent that it may differ depending on the brain region and even in different synapses from the same neuron (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). For example, as discussed above, in cortical neurons EphB2 is in spine synapses and ephrin-B3 seems to be in shaft synapses. B-type ephrins are presynaptic in area CA3 of the mouse hippocampus and the *Xenopus* optic tectum but postsynaptic in area CA1 of the hippocampus.

EphB receptors are also postsynaptic in area CA1, and it is unclear whether they are in the same dendritic spines as B-type ephrins or in mutually exclusive subpopulations of spines. To complicate matters further, EphA4, which is the Eph receptor most highly expressed in the adult hippocampus and can interact with all ephrins, has been detected by electron microscopy not only in spines but also in presynaptic terminals (Tremblay et al., 2007).

Electrophysiological measurements using hippocampal slices have demonstrated that the Eph system plays a role in paradigms of activity-dependent synaptic plasticity that model learning and memory (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). These include long-term potentiation (LTP), where high-frequency electrical stimulation increases synaptic strength; long-term depression (LTD), where low-frequency stimulation reduces synaptic strength; and depotentiation, where low-frequency stimulation reverses the effects of LTP. In an initial study, ephrin-A5 Fc treatment caused an LTP-like effect whereas EphA Fc inhibited LTP (Yamaguchi and Pasquale, 2004). The mechanisms underlying these effects, which likely depend on EphA4 and possibly other less abundant EphA receptors, remain unclear. EphA4 in the dendritic spines of hippocampal neurons has been implicated in communication with astrocytes, which express ephrin-A3 on their perisynaptic processes. EphA4 activation by ephrin has been recently shown to inhibit the Rap1 and Rap2 GTPases and integrin activity and to promote RhoA and PLCγ activity (Figure 1), causing spine retraction and synapse loss as well as changes in spine shape (Bourgin et al., 2007; Fu et al., 2007; Richter et al., 2007; Zhou et al., 2007). These effects of EphA4 forward signaling would be predicted to affect synaptic plasticity, perhaps enabling an influence of astrocytes on synaptic function.

Electrophysiological measurements have also shown reduced LTP and LTD at hippocampal synapses of area CA1 in EphB2 and EphA4 knockout mice, although basal synaptic transmission was normal (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). For both receptors, however, knockin mutants lacking the kinase domain rescued the defects, suggesting that EphB2 and EphA4 forward signaling is not required for these forms of synaptic plasticity. Because synaptic plasticity in area CA1 depends on postsynaptic mechanisms, EphB2 may regulate plasticity by associating with NMDA ion channel receptors and by promoting their synaptic localization. Alternatively, EphB2 and/or EphA4 may stimulate reverse signaling through postsynaptic ephrins.

Studies with mutant mice have also shown that reverse signaling by postsynaptic ephrin-B2 plays an essential role in synaptic plasticity in area CA1 of the hippocampus (Bouzioukh et al., 2007; Yamaguchi and Pasquale, 2004). The PDZ domain-binding site of ephrin-B2 is required for LTP, LTD, and depotentiation, whereas the tyrosine phosphorylation sites are only important for LTP. The involvement of ephrin-B3 in synaptic plasticity in area CA1 remains to be clarified because different groups have reported either defective or normal LTP in ephrin-B3 knockout mice (Dalva et al., 2007). Reverse signaling by presynaptic B-type ephrins has been implicated in the regulation of LTP in area CA3, which depends on presynaptic mechanisms. This effect is due to *trans*-synaptic bidirectional communication with postsynaptic EphB2, possibly regulating presynaptic vesicle release. Similarly, presynaptic ephrin-B signaling has been recently shown to enhance presynaptic glutamate release and postsynaptic glutamate responsiveness in developing *Xenopus* retinotectal synapses, where EphB2 is also localized postsynaptically (Lim et al., 2008).

Given the involvement of the Eph system in the regulation of dendritic spine morphology and synaptic plasticity, its dysfunction would be predicted to cause learning and memory deficits. Indeed, some Eph/ephrin mutations and hippocampal infusion of Eph/ephrin Fc fusion proteins have been shown to affect learning and memory performance in mice (Dalva et al., 2007; Yamaguchi and Pasquale, 2004). It will be interesting to investigate whether Eph/ephrin dysfunction may cause some forms of mental retardation and the accompanying dendritic spine abnormalities, and whether downregulation of EphB2 cell-surface clusters by soluble amyloid β protein has a role in the synapse/spine degeneration and memory loss characteristic of Alzheimer's disease (Lacor et al., 2007). Repeated exposure to drugs of abuse also causes long-lasting changes in the neuronal circuits of certain brain regions, including hippocampus and cortex, and alterations in Eph receptor/ephrin expression might contribute to some of these effects (Bahi and Dreyer, 2005). Better understanding of how Eph bidirectional signaling regulates synaptic plasticity may suggest new strategies to help counteract the cognitive and behavioral problems associated with mental retardation, aging, or drug addiction.

Repair after Injury

Upregulation of multiple Eph receptors and ephrins has been detected at sites of nervous system injury (Du et al., 2007). In some cases, developmental expression patterns are recapitulated. In others, new patterns develop under the regulation of cytokines, hypoxia, and other factors present at sites of injury. Some of the Eph receptors/ephrins expressed in neural cells may provide guidance cues enabling the re-establishment of appropriate connections, but they may also hinder proper axon regrowth through their repulsive signaling (Wu et al., 2007). Eph receptors and ephrins present in inflammatory cells and meningeal fibroblasts that infiltrate the injury site can also engage in bidirectional signaling with Eph proteins upregulated in neural cells, with consequences for regeneration. For example, EphB3 expressed in the macrophages recruited to the injured mouse optic nerve promotes sprouting of damaged retinal axons, which express ephrin-B3 (Liu et al., 2006). Furthermore, the interplay between EphB2 expressed in invading meningeal fibroblasts and ephrin-B2 expressed in reactive astrocytes after rat spinal cord transection appears to promote the segregation of the two cell types and the formation of the glial scar and surrounding basal lamina.

The EphA4 receptor is emerging as an inhibitor of nerve regeneration. After lesions to the spinal cord, this receptor accumulates in both damaged corticospinal axons and reactive astrocytes (Du et al., 2007; Fabes et al., 2007). Analysis of EphA4 knockout mice and infusion of an EphA4

antagonistic peptide in the intrathecal space surrounding the rat spinal cord suggest that EphA4 forward signaling plays a role in the axon retraction that occurs after lesion and also hinders subsequent axon sprouting/regeneration and behavioral recovery. This could be due to interaction of axonal EphA4 with both ephrin-B2 expressed in reactive astrocytes and ephrin-B3 expressed in myelin. EphA4 in reactive astrocytes may also play a role in the formation of the glial scar, which forms a barrier impeding axon regeneration. According to these still preliminary but intriguing studies, strategies to inhibit EphA4 function promise to be beneficial for the treatment of spinal cord injury. More extensive studies on the involvement of the Eph system in different regions of the central nervous system after various types of injury will help identify possible Eph-based strategies to improve recovery.

Despite the progress over many years in elucidating the activities of Eph bidirectional signaling in neural development, plasticity, and repair, new exciting roles continue to be discovered for these molecules. That a single Eph receptor, or ephrin, can affect multiple processes through different signaling mechanisms underscores how effectively the complexity and versatility of the Eph system have been exploited in the nervous system.

Immune Function

Many Eph receptors and ephrins are expressed in lymphoid organs and lymphocytes, suggesting that they have immunoregulatory properties (Wu and Luo, 2005). For example, the Eph system seems to play a role in immune processes where cell contact-dependent communication is critical, such as the development of thymocytes into mature T cells within the thymus and the subsequent differentiation of activated T cells into effector cells in the periphery.

Several studies have shown that perturbing Eph-ephrin interactions in thymic organ culture with Eph or ephrin Fc fusion proteins interferes with thymocyte survival and maturation (Alfaro et al., 2007; Munoz et al., 2006; Wu and Luo, 2005). Defects in thymocyte maturation have also been observed in EphA4 knockout mice, which have greatly decreased numbers of peripheral T cells. These defects appear to result from abnormal development of the stromal cells of the thymic cortex, which express EphA4 and support thymocyte survival and maturation. Preliminary observations suggest that EphB2 and EphB3 knockout mice also have a disorganized thymic architecture and decreased numbers of thymocytes. These findings suggest that the Eph system is important for the structural organization of the thymus and for guiding the movement of thymocytes through the different thymic compartments that support their gradual maturation into T cells.

Other studies have shown that the Eph receptors modulate responses mediated by the T cell receptor (TCR) and may represent a class of costimulatory receptors. EphB6 is the Eph receptor whose function in immune regulation has been best characterized (Wu and Luo, 2005). This receptor is highly expressed in the thymus, where it is present in a substantial fraction of thymocytes, particularly those double positive for CD4 and CD8. EphB6 has also been detected in a fraction of peripheral $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells, where its levels appear to be dynamically regulated by rapid synthesis and removal. Although EphB6 lacks kinase activity, stimulation of T cells with anti-EphB6 antibodies or ephrin-B ligands leads to increased tyrosine phosphorylation and intracellular signaling. EphB6 phosphorylation may occur through association with coexpressed EphB receptors, such as EphB1 and possibly EphB4. Several cytoplasmic signaling molecules known to participate in TCR signaling, such as the adaptor and ubiquitin ligase Cbl, associate with EphB6 and have been implicated in its effects.

There is substantial evidence that EphB receptors modulate T cell responses (Alfaro et al., 2007; Wu and Luo, 2005; Yu et al., 2006). First, these receptors cluster with activated T cell receptors in aggregated lipid rafts. Second, clustering of EphB receptors with immobilized anti-EphB6 antibodies or ephrin-B Fc ligands lowers the activation threshold of T cells responding to suboptimal TCR ligation. EphB activation also promotes T cell proliferation, production of interferon γ (but not interleukins 2 and 4), and cytotoxic T cell activity. These effects involve upregulation of the p38 and p42/44 MAP kinases. Third, EphB6-negative T cells purified from human peripheral blood or from the spleen of EphB6 knockout mice show impaired TCR signaling, proliferation, and cytokine secretion in vitro. Fourth, the EphB6 knockout mice show impaired cellular immune responses despite having normal T cell numbers. Thus, EphB receptor ligation enhances the effects of weak TCR signaling, suggesting that EphB receptors promote positive thymocyte selection and T cell responses to antigen-presenting cells. On the other hand, in thymocytes and Jurkat T cells EphB receptor signaling has also been reported to blunt the effects of high TCR signaling, such as interleukin-2 secretion and induction of apoptosis. Hence, EphB receptor ligation might also inhibit the effects of strong TCR signaling, such as the negative selection of self-reactive thymocytes.

Physiologically, EphB receptors in T cells are likely activated through interactions with ephrin-B ligands expressed by other T cells as well as other cell types, such as thymic epithelial cells and antigen-presenting cells (Wu and Luo, 2005). Interestingly, these Eph interactions may facilitate T cell responses in lymphoid organs, where T cells and antigen-presenting cells have sustained contact to promote differentiation of naive T cells into effectors.

EphA receptors and A-type ephrins are also expressed in thymocytes and T cells (Freywald et al., 2006; Wu and Luo, 2005) and have also been reported to modulate TCR signaling. For example, stimulation of $CD4^+$ $CD8^+$ double-positive thymocytes with ephrin-A1 Fc inhibits interleukin-2 secretion and apoptosis induced by strong TCR activation. This suggests that EphA receptors modulate negative selection of self-reactive thymocytes, which depends on apoptosis triggered by strong TCR stimulation. Ephrin-A1 is also expressed in $CD4^+$ helper T cells, where it may have a functional effect through reverse signaling because its ligation with antibodies has been reported to suppress TCR responses. Furthermore, the EphA system has been proposed to modulate

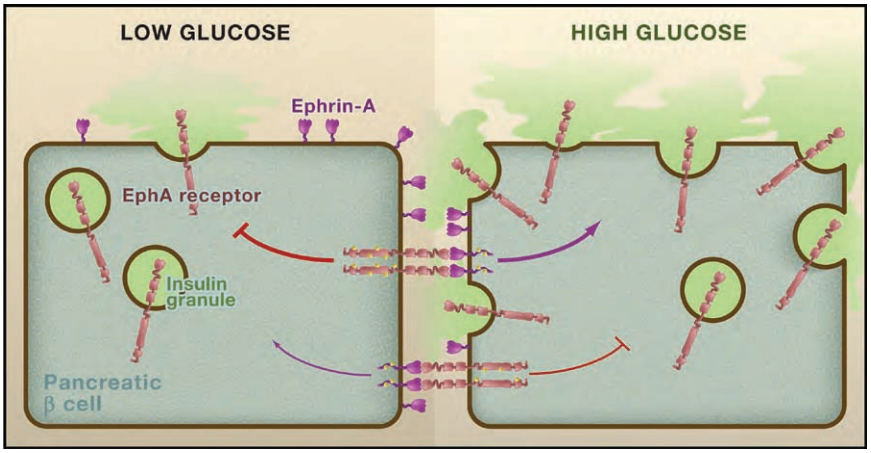

Figure 3. EphA-Ephrin-A Bidirectional Signaling and Insulin Secretion

When glucose levels are low, forward signaling predominates inhibiting insulin secretion; when glucose levels are high, reverse signaling predominates promoting insulin secretion. Ephrin-A molecules are mainly on the cell surface whereas Eph receptor molecules are also in the secretory granules. Thicker lines indicate stronger signals; yellow circles, tyrosine phosphorylation.

thymocyte and T cell migratory responses to chemokines (such as SDF1) and integrin-dependent adhesion, which guide thymocyte movements within the thymus and T cell trafficking between the blood, lymphoid tissues, and sites of extravasation (Hjorthaug and Aasheim, 2007; Sharfe et al., 2008; Wu and Luo, 2005). Signaling molecules that have been implicated in EphA-dependent regulation of T cell migration include the cytoplasmic tyrosine kinases Lck and Pyk2, the exchange factor Vav1, and Rho family GTPases. However, more work is needed to establish the physiological significance of the EphA-dependent chemotactic and adhesive responses observed in vitro.

Eph receptors and ephrins are also expressed in B lymphocytes, but their effects in these cells have not been characterized (Aasheim et al., 2000; Nakanishi et al., 2007). Clearly, more work is needed to refine our knowledge of Eph bidirectional signaling in the immune system. As in other organs, the role of these molecules is likely to be complex and involve the coordinated activities of different Eph receptors and ephrins that have intertwined and partially overlapping functions. Careful expression studies and evaluation of immunological defects in compound Eph and ephrin conditional knockout mice will be particularly useful for dissecting these roles. It will also be important to determine whether defects in Eph function contribute to immunological disorders and hematopoietic malignancies where Eph proteins are highly expressed (Nakanishi et al., 2007).

Glucose Homeostasis and Diabetes

The β cells in the pancreas adjust their secretion of insulin in response to glucose levels in the blood in order to maintain glucose homeostasis in the body. Communication between β cells clustered in pancreatic islets has long been known to modulate insulin secretion, but the underlying molecular mechanisms were unknown. A recent study using cultured cells and mouse models shows that β cells communicate via EphA receptors and ephrin-A ligands (Konstantinova et al., 2007). Remarkably, EphA forward signaling (which inhibits insulin secretion) and ephrin-A reverse signaling (which enhances insulin secretion) can be differentially regulated in pancreatic cells (Figure 3). When glucose is low, EphA forward signaling predominates, decreasing basal insulin secretion. Glucose causes EphA receptor dephosphorylation, leading to downregulation of EphA forward signaling without inhibition of ephrin-A reverse signaling. Thus, reverse signaling predominates when glucose is high, increasing insulin secretion. A further twist is that although ephrin-A ligands are mainly localized on the plasma membrane, EphA receptors are also in the intracellular insulin secretory granules. This suggests that EphA levels on the plasma membrane, and therefore EphA-ephrin-A complexes, increase upon insulin release. This causes a negative feedback loop that limits insulin secretion through increased EphA signaling when glucose levels are low and a positive feedback loop that potentiates secretion through increased ephrin-A signaling when glucose levels are high (Figure 3).

Although further studies will be required to fully elucidate the signaling pathways underlying these effects, some evidence suggests that the opposite effects of EphA and ephrin-A signaling depend on differential regulation of Rac1 GTPase activity and actin filament assembly as well as gap junction communication. A number of intriguing questions also remain. First, do EphB receptors and ephrin-B ligands—which are also expressed in pancreatic β cells—contribute to the regulation of glucose homeostasis or have other functions? Second, do these results in the pancreas reveal a general mechanism by which Eph receptors and ephrins regulate exocytosis in other secretory systems? Third, do the Eph-dependent defects in insulin secretion play a role in type 2 diabetes and might the ability of the EphA/ephrin-A system to affect insulin release be exploited in the treatment of diabetes?

Bone Maintenance and Bone Remodeling Diseases

Developmental deficiencies in EphB/ephrin-B signaling can cause skeletal malformations. These include cleft palate, defective development of the skull vault, craniosynostosis, and other bone abnormalities observed in EphB2/EphB3 and ephrin-B1 mutant mice and in individuals harboring ephrin-B1 mutations that cause the X-linked developmental disorder craniofrontonasal syndrome (Davy et al., 2006; Pasquale, 2005). Interestingly, mosaic ephrin-B1 expression in calvarial osteoblast precursors—due to random X chromosome inactivation in ephrin-B1 heterozygous females—causes abnormal cell sorting leading to defects in bone development. Genetic and other evidence supports a model

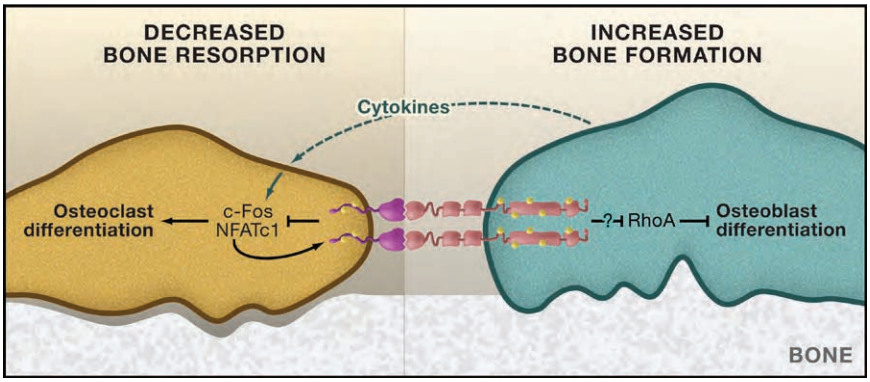

Figure 4. EphB-Ephrin-B Bidirectional Signaling in Bone Formation

Osteoblasts secrete cytokines that upregulate ephrin-B2 in osteoclast precursors. Ephrin-B ligands in osteoclasts interact with EphB receptors in osteoblasts generating bidirectional signals that inhibit osteoclast differentiation and promote osteoblast differentiation.

in which EphB-ephrin-B1 bidirectional signaling at the ectopic boundaries that form between ephrin-B1 positive and negative osteoblast precursors leads to impaired gap junction communication, which inhibits osteoblast differentiation and delays ossification of developing calvarial bones.

Besides these developmental roles, EphB-ephrin-B bidirectional signaling between osteoblasts and osteoclasts has been implicated in the regulation of bone homeostasis in the adult (Zhao et al., 2006). Bones continue to be remodeled throughout life, a process controlled by dynamic reciprocal communication between osteoclasts, which degrade bone, and osteoblasts, which form bone. Gain- and loss-of-function experiments in culture have shown that cytokines produced by osteoblasts activate the transcription factors c-Fos and NFATc1 in osteoclast precursors. This promotes osteoclast differentiation and also increases ephrin-B2 expression (Figure 4). Several Eph receptors present in osteoblasts can stimulate ephrin-B reverse signaling in osteoclasts, which represses osteoclast differentiation through a negative feedback loop that inhibits c-Fos and NFATc1 and requires the ephrin PDZ domain-binding site.

The communication between osteoclasts and osteoblasts is bidirectional and forward signaling by EphB4—and possibly other coexpressed Eph receptors—promotes the differentiation of osteoblasts, which deposit new bone at sites of resorption by osteoclasts (Figure 4). The Eph forward signaling pathway responsible for osteoblast differentiation may involve RhoA inactivation. Hence, cell contact-dependent communication between Eph receptors and ephrins limits osteoclast differentiation and enhances osteoblast differentiation, inducing a shift from bone resorption to bone formation. Indeed, transgenic overexpression of EphB4 in osteoblasts has been shown to increase bone mass in mice.

These findings suggest that interventions targeting the EphB system may be helpful in the prevention and treatment of bone remodeling diseases, such as osteoporosis and osteopetrosis. It will be important, however, to elucidate the role of bidirectional signaling between osteoblasts, which in addition to EphB receptors also express B-type ephrins. Another unresolved issue with possible therapeutic implications is whether Eph-ephrin interactions between cancer cells and osteoblasts or osteoclasts may play a role in bone metastatic disease.

Intestinal Homeostasis

The intestine is lined by a monolayer of epithelial cells that control the absorbance of nutrients and the secretion of protective mucus and antimicrobial agents. The intestinal epithelium undergoes continuous self-renewal throughout life, and homeostasis is maintained by the balance of cell proliferation, differentiation, and apoptosis. A recent study has shown that a few cycling cells located at the bottom of invaginations called crypts can generate all intestinal epithelial lineages and therefore likely represent the long sought-after intestinal stem cells (Barker et al., 2007). The stem cells give rise to rapidly proliferating transit-amplifying cells, which differentiate while migrating toward the top of the crypts. In the small intestine, epithelial cells continue to migrate toward the tips of protrusions called villi, where they die and are shed into the intestinal lumen.

The canonical Wnt/β-catenin/Tcf signaling pathway is a critical regulator of homeostasis in the intestinal epithelium, in part through its ability to promote the transcription of EphB receptors and inhibit that of ephrin-B ligands (Clevers and Batlle, 2006). As the newly generated epithelial cells migrate, they gradually lose EphB expression and acquire ephrin-B expression as they move away from the source of Wnt secreted by surrounding mesenchymal cells at the bottom of the crypts. This creates countergradients of EphB and ephrin-B expression along the crypt axis, with high EphB expression at the bottom of the crypts and high ephrin-B expression at the top and in the villi. A population of secretory cells in the small intestine, called Paneth cells, also undergoes renewal but remains interspersed with the stem cells at the bottom of the crypts. Unlike other intestinal epithelial cells, Paneth cells can differentiate when Wnt levels are high. They also maintain high EphB3 expression after differentiation, which is important for their localization.

Analysis of EphB2/EphB3 and ephrin-B1 knockout mice, and knockin mice expressing a dominant-negative form of EphB2 replacing the wild-type receptor, has shown that EphB-dependent repulsive signaling restricts intermingling of the proliferating and differentiated cells (Clevers and Batlle, 2006; Cortina et al., 2007). Interestingly, crosstalk with E-cadherin appears to play a crucial role (Figure 2). EphB forward signaling promotes E-cadherin-mediated cell adhesion in colorectal cancer cells, and E-cadherin is required for the in vitro sorting of EphB- and ephrin-B-expressing cells into separate cell clusters.

Perturbation of EphB forward signaling in the mouse through genetic manipulations or administration of soluble forms of the ephrin-B2 or EphB2 extracellular domains has also implicated the EphB system in intestinal epithelial cell

proliferation (Holmberg et al., 2006). Cell proliferation was decreased on the sides of the crypts and not at the bottom, suggesting that the EphB system promotes the proliferation of transit-amplifying cells.

It will be important to also examine the role of the EphA/ephrin-A system in intestinal homeostasis because uneven mRNA expression along the crypts of the colon has also been reported for several EphA receptors and ephrin-A1 (Kosinski et al., 2007). EphA2 and ephrin-A1 have also been suggested to regulate epithelial barrier function in the intestine (Rosenberg et al., 1997). Future studies to explore whether Eph receptors and ephrins may play a role in intestinal diseases, such as inflammatory bowel disease, or in the restoration of the injured intestinal epithelium (Hafner et al., 2005; Rosenberg et al., 1997) will provide a more complete understanding of the Eph system in intestinal homeostasis and disease. The EphB system has also been implicated in colorectal cancer (see next section). The Eph bidirectional signaling pathways in normal and transformed intestinal epithelial cells also await a comprehensive investigation.

Cancer

Besides their expression in normal tissues, Eph receptors and/or ephrins are present, and often upregulated, in essentially all types of cancer cells (Ireton and Chen, 2005; Noren and Pasquale, 2007). In many cases this may be due to oncogenic signaling pathways, hypoxia, or inflammatory cytokines. For example, the Wnt/β-catenin/Tcf pathway promotes EphB expression in colorectal cancer cells and the Ras-MAP kinase pathway promotes EphA2 expression in breast cancer cells. Interestingly, activation of these two pathways also results in ephrin downregulation and, as a consequence, low Eph receptor activation. Indeed, Eph receptor forward signaling does not necessarily aid the tumorigenic process. Tumor suppressor activities have been reported for Eph signaling in colorectal, breast, prostate, and skin cancer cells both in vitro and in vivo. However, the decreased tumorigenicity of cancer cells in which Eph receptor expression was experimentally decreased suggests that these receptors can also have tumor-promoting effects. The role of ephrin reverse signaling in cancer cells is poorly characterized, although several ephrins have been reported to promote cell transformation and cancer cell migration/invasion (Campbell et al., 2006; Meyer et al., 2005; Tanaka et al., 2007). To complicate matters further, the Eph system is also operational in the tumor microenvironment. The effects of Eph-ephrin bidirectional signaling have been mostly studied in tumor endothelial cells, whereas information on other types of tumor stromal cells is very limited. In order to design rational strategies to target the Eph system for cancer therapy, we need to further elucidate how Eph receptors and ephrins influence the behavior of cancer cells, cancer stromal cells, and also cancer stem cells. Below we discuss work on several cancers, which exemplifies our current understanding of the Eph system in oncogenic transformation.

Colorectal Cancer

The same signaling proteins that control physiological self-renewal in the intestine can also initiate malignant transformation when mutations subvert their activity. Thus, constitutive activation of the Wnt/β-catenin/Tcf pathway leads to the formation of adenomas and colorectal cancer (Clevers and Batlle, 2006). As in the normal intestine, the pathway also upregulates EphB expression in the early stages of tumorigenesis. Despite their reported ability to promote proliferation in the intestinal epithelium, the EphB receptors appear to have a tumor suppressor role in colorectal cancer. Indeed, in advanced human colorectal cancers expression of different EphB receptors is lost in a large fraction of the tumor cells, and there is strong association of tumor histological grade and patient survival with EphB silencing (Batlle et al., 2005). Intriguingly, hypoxia may explain the coordinated downregulation of multiple EphB receptors in advanced cancers because hypoxia-inducible factor-1 can compete with Tcf-4 for binding to nuclear β-catenin, leading to silencing of Tcf-4 target genes (Kaidi et al., 2007).

Reduced EphB activity accelerates the progression of colorectal cancer. This is supported by studies with the $Apc^{Min/+}$ mouse model, where poorly differentiated and aggressive colorectal adenocarcinomas develop in mice lacking EphB3 or ephrin-B1 and in mice expressing dominant-negative EphB2 but not in control mice (Batlle et al., 2005; Cortina et al., 2007). A possible mechanism inhibiting the expansion of EphB-positive tumor cells involves E-cadherin-dependent spatial restriction by surrounding epithelial cells that express ephrin-B ligands. The involvement of the EphA/ephrin-A system in colorectal cancer remains to be investigated using mouse models, to follow up on cell culture studies suggesting oncogenic effects of coexpressed EphA2 and ephrin-A1 (Wimmer-Kleikamp and Lackmann, 2005).

Breast Cancer

EphA2 and EphB4 are the Eph receptors most extensively studied in breast cancer, although our understanding of their activities is far from complete (Ireton and Chen, 2005; Macrae et al., 2005; Noren and Pasquale, 2007). Both receptors are widely expressed but poorly tyrosine phosphorylated in human breast cancer cell lines, suggesting a low level of ephrin-dependent activation. Indeed, the levels of ephrin-B2—the preferred ligand for EphB4—are low in these cell lines, and high EphA2 expression also correlates with low ephrin-A expression. Intriguingly, even when ephrin-A1 is present, its ability to activate EphA2 may be impaired in breast cancer cells that lack E-cadherin. These data suggest that if EphA2 and EphB4 have oncogenic activity in human breast cancer cell lines, this activity must be either independent of ephrin stimulation or manifest itself when ephrin stimulation is low.

Overexpression of EphA2 in a human mammary epithelial cell line has been shown to cause oncogenic transformation (Ireton and Chen, 2005; Noren and Pasquale, 2007). Despite the fact that EphA2 was poorly tyrosine phosphorylated, the overexpressing cells acquired the ability to grow in soft agar and form tumors in mice. Furthermore, they had decreased estrogen dependence and sensitivity to the drug tamoxifen. On the other hand, EphA2 knockdown by RNA interference or with antisense oligonucleotides has been shown to inhibit the tumorigenicity of several types of cancer cells, including a breast cancer cell line. Similarly, EphB4 knockdown inhibited breast cancer cell survival, migration, and invasion, and also tumor growth in a mouse xenograft model.

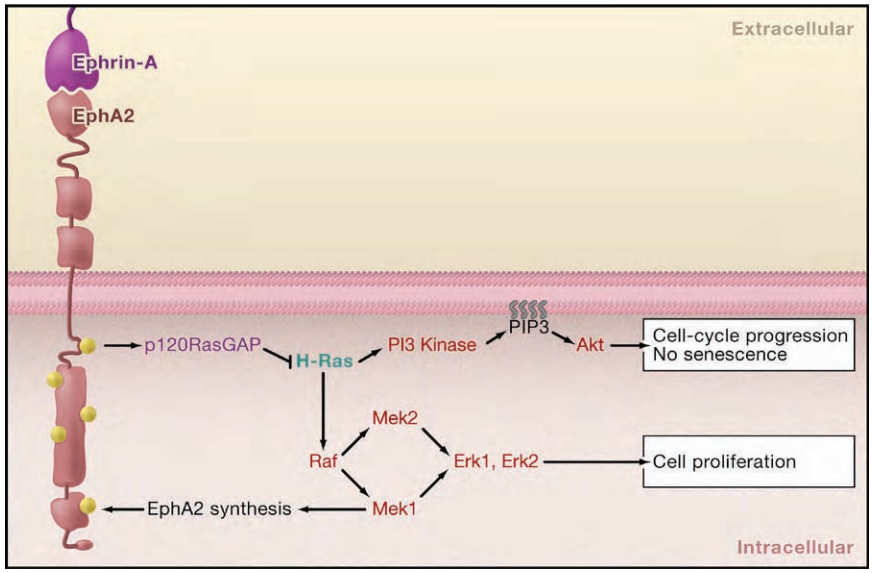

Figure 5. EphA2, Cell-Cycle Arrest, and Cellular Senescence

Raf-activating mutations upregulate the levels of EphA2, which may contribute to cell-cycle arrest and senescence through inhibition of H-Ras-PI3 kinase-Akt. In cells without activated Raf, EphA2 also inhibits the MAP kinase pathway.

The mechanisms underlying these oncogenic effects of Eph receptors that appear to be poorly activated are unclear. Some evidence suggests that ephrin-independent crosstalk with oncogenic signaling pathways may be involved. For example, EphA2 has been found to enhance tumor cell proliferation and motility in cells overexpressing EGF receptor family members, an activity that likely contributes to tumorigenesis and metastatic progression in a mouse ErbB2 mammary adenocarcinoma model (Brantley-Sieders et al., 2008; Larsen et al., 2007). The Eph receptors might also serve as scaffolds for constitutively associated signaling proteins, somehow affecting their localization and signaling ability to promote cell transformation. One study has shown that when transformed by EphA2 overexpression, mammary epithelial cells deposit more fibronectin, which plays a role in their survival (Hu et al., 2004). Oncogenic signaling pathways that may be activated by low ephrin levels could also be responsible for the tumorigenic effects of EphA2 and EphB4 in breast cancer cells.

Low versus high Eph forward signaling might have opposite effects on tumorigenicity, as has been shown for other cellular properties (Pasquale, 2005; Poliakov et al., 2004). EphA2 dephosphorylation by the low-molecular-weight phosphotyrosine phosphatase has been shown to promote mammary epithelial cell transformation, presumably by inhibiting EphA2 forward signaling (Noren and Pasquale, 2007; Wimmer-Kleikamp and Lackmann, 2005). Furthermore, EphA2 and EphB4 activation with soluble ephrin ligands or activating antibodies decreases the malignant properties of human breast cancer cell lines. Activation of EphA2 inhibited growth in soft agar, fibronectin deposition, cell survival, and tumor growth in a breast cancer xenograft model (Ireton and Chen, 2005). Inhibition of Ras activity downstream of EphA2 likely plays an important role in these tumor suppressor effects by inhibiting downstream MAP kinases and possibly also the PI3 kinase-Akt pathway (Figure 5) (Menges and McCance, 2007). EphB4 activation also inhibits breast cancer cell growth and migration (Noren and Pasquale, 2007). These effects involve activation of Abl family tyrosine kinases and tyrosine phosphorylation of the adaptor protein Crk, likely inhibiting Rac activity (Figure 1B). Curiously, high levels of ephrin stimulation produce effects similar to EphA2 or EphB4 knockdown in cultured breast cancer cells. Further studies are needed to elucidate the mechanisms underlying the antioncogenic effects of ephrin stimulation versus downregulation of Eph receptor expression.

A possible working hypothesis is that high levels of ephrin-dependent EphA2 and EphB4 forward signaling suppress tumorigenesis whereas low levels of forward signaling or crosstalk with oncogenic signaling pathways promote tumorigenicity. However, in contrast to its tumor suppressor effects in human breast cancer cells, EphA2 kinase activity appears to promote tumorigenesis in mouse 4T1 mammary tumor cells, which express ephrin-A1 (Brantley-Sieders et al., 2006). In these cells, EphA2 kinase activity promotes VEGF secretion, RhoA activation, and cell motility in vitro as well as tumor growth and metastasis in mouse models. EphA2 is also tyrosine phosphorylated and coexpressed with ephrin-A1 in other types of cancer cells, including malignant melanoma cells, suggesting divergent roles for EphA2 in cell transformation depending on the cellular context (Ireton and Chen, 2005). Perhaps, cancer cells that endogenously express highly activated Eph receptors have evolved mechanisms to neutralize their tumor suppressor signals. For example, Ras- and Raf-activating mutations could counteract some of the antioncogenic effects of activated EphA2 (Figure 5) (Menges and McCance, 2007).

Skin Cancer and Melanoma

The most common types of skin cancer are derived from either melanocytes or keratinocytes, and EphA2 appears to have different effects in the two types of cancer cells. In melanoma, ephrin-A1-mediated activation of EphA2 and possibly other EphA receptors promotes proliferation (Easty and Bennett, 2000; Hess et al., 2007). Intriguingly, EphA2 has also been found to associate with vascular endothelial cadherin and promote the formation of blood vessel-like structures by malignant melanoma cells, a role similar to that of EphA2 in tumor endothelial cells (see below). In contrast, a recent study has shown that susceptibility to chemically induced keratinocyte transformation is enhanced in EphA2 knockout mice (Guo et al., 2006). Furthermore, despite the observed upregulation of EphA2 in mouse as well as human keratinocyte-derived skin carcinomas, the tumors lacking EphA2 grow faster and are more invasive.

Similar to the EphB/ephrin-B interplay in colorectal cancer, ephrin-A1 expression in the surrounding skin appears to restrict expansion of the EphA2-positive tumor cells. Inhibition of Ras-dependent pathways may explain these tumor suppressor effects of EphA2.

Bidirectional signaling through other Eph receptors and ephrins can also have diverse effects on melanoma malignancy. For example, EphB4 activation by coexpressed ephrin-B2 in the aggressive SW1 mouse melanoma cell line promotes RhoA activation, leading to increased ameboid migration (Noren and Pasquale, 2007). In contrast, EphB4 activation with ephrin-B2 Fc in the human MDA-MB-435 cell line (which has low endogenous ephrin-B2 expression) inhibits proliferation, survival, migration, and invasion in vitro as well as tumor growth in a mouse xenograft model through a pathway involving Abl and Crk. It should be noted that a recent study provides strong evidence that the currently available stocks of MDA-MB-435 cells, which were previously believed to be of breast cancer origin, are instead derived from a melanoma line (Rae et al., 2007).

In addition to promoting EphB signaling, endogenous ephrin-B2 expressed in melanoma cells has also been found to associate with β1-integrins and promote cell adhesion and migration, suggesting a role in tumor progression through reverse signaling and crosstalk with integrins (Figure 2) (Meyer et al., 2005). The EphA4 receptor is expressed in melanocytes but downregulated in aggressive melanoma cells, suggesting that EphA4 has a role as a melanoma tumor suppressor (Easty and Bennett, 2000). EphB6 is also downregulated during melanoma progression, but this receptor lacks kinase activity and thus may function differently from other Eph receptors (Hafner et al., 2003).

Tumor Angiogenesis

Besides being expressed in cancer cells, Eph receptors and ephrins are also present in the tumor vasculature, where they promote angiogenesis (Brantley-Sieders and Chen, 2004; Heroult et al., 2006; Noren and Pasquale, 2007). Because blood vessels are critical for tumor growth and metastasis, this is an important aspect of the oncogenic effects of Eph-ephrin bidirectional signaling. The main roles in tumor angiogenesis have so far been attributed to EphA2 forward signaling and ephrin-B2 reverse signaling based on a series of in vitro and in vivo experiments with mouse tumor models, including analysis of angiogenesis in EphA2 knockout mice. Interestingly, EphA2 is not expressed in the embryonic vasculature or the adult quiescent vasculature. Interaction with ephrin-A1 present in tumor endothelial cells as well as tumor cells is responsible for activating endothelial EphA2. Signaling effectors that have been implicated in the angiogenic activity of EphA2 include PI3 kinase, Vav guanine nucleotide exchange factors, and Rac1 (Figure 1B). Activation of these effectors presumably impacts the actin cytoskeleton, thus regulating endothelial cell shape and migration. Interestingly, EphA2 appears to be required for VEGF-induced endothelial cell migration and assembly into capillary-like structures (Chen et al., 2006).

Ephrin-B2 is also widely expressed in the vasculature of many tumors, which is not surprising given that this ephrin is found in the embryonic arterial vasculature and its expression in endothelial cells is upregulated by hypoxia and VEGF (Brantley-Sieders and Chen, 2004; Heroult et al., 2006; Noren and Pasquale, 2007). Ephrin-B2 reverse signaling can be stimulated by interaction with EphB4 expressed in the tumor vasculature and in tumor cells. Indeed, increased levels of the EphB4 extracellular portion on the surface of a cancer cell line have been shown to increase tumor growth through effects on the vasculature. EphB4 activation by ephrin-B2 in circulating endothelial progenitor cells also increases their recruitment to sites of neovascularization through selectin-mediated adhesion (Foubert et al., 2007). It will be interesting to investigate whether this also contributes to tumor neovascularization.

Given the divergent effects of Eph receptors and ephrins in cancer cells, Eph-based anticancer therapies involving vascular targeting seem the most straightforward. Indeed, various approaches to interfere with EphA2-ephrin-A or EphB-ephrin-B2 binding using soluble Eph extracellular domains have consistently resulted in inhibition of tumor growth in various mouse models (Heroult et al., 2006; Ireton and Chen, 2005; Noren and Pasquale, 2007; Wimmer-Kleikamp and Lackmann, 2005). However, targeting the Eph system will also affect the tumor cells, likely with variable outcomes depending on the tumor type. Ultimately, the efficacy of each Eph-based targeting strategy will have to be evaluated empirically in appropriate in vivo tumor models.

Cancer Stem Cells

An emerging theme in cancer therapy is the possible importance of targeting the "cancer stem cells," which are the cells that can repopulate the tumor and cause recurrence even when most of the tumor mass has been eliminated. Because Eph receptors/ephrins are expressed in various other types of stem cells, they are also likely to be present in cancer stem cells (Pasquale, 2005). However, characterization of the Eph system in stem cells is still at an early stage. Positive as well as negative effects on proliferation, apoptosis, and differentiation have been reported depending on the Eph/ephrin involved and the type of stem cell. An area of particular interest is the role of Eph-ephrin bidirectional signaling in the communication between stem cells and their supporting niche cells. Intriguingly, a recent study has implicated Eph receptor-dependent inhibition of the Ras-MAP kinase pathway in the asymmetric division of at least two different precursor cells in the ascidian embryo (Picco et al., 2007; Shi and Levine, 2008). It was shown that contact with asymmetrically localized ephrin-expressing neighboring cells triggers polarized Eph receptor activity, driving specification of one of the two daughter cells to a neural rather than notochord fate or to a mesodermal rather than an endodermal fate. It will be interesting to investigate whether Eph-ephrin interactions with niche cells might have a similar role in the self-renewal versus differentiation choice during asymmetric stem cell division. Knowing the effects of Eph-ephrin signaling in cancer stem cells will likely be important in deciding how to target these molecules for anticancer therapy.

Henipavirus Infection

It was recently discovered that ephrin-B2 and ephrin-B3 serve as the cell entry receptors for Nipah and Hendra viruses, two emerging paramyxoviruses comprising the newly defined

Henipavirus genus (Bonaparte et al., 2005; Negrete et al., 2005, 2006). Although the natural host for henipaviruses is the fruit bat, outbreaks in farm animals and transmission to humans have repeatedly occurred in recent years. The high evolutionary conservation of the ephrins explains the ability of Nipah and Hendra viruses to infect a wide range of animal species. In humans, these viruses are highly lethal and are classified as category 4 containment pathogens. The tissue distribution of ephrin-B2 in the vascular system and both ephrin-B2 and ephrin-B3 in the nervous system are consistent with the tissue tropism of the viruses. Both Nipah and Hendra viruses bind to the same region of ephrin-B2 and ephrin-B3 that also mediates high-affinity binding to EphB receptors. It will therefore be interesting to determine whether disruption of EphB/ephrin-B function, or activation of reverse signals following ephrin-B clustering by the tetrameric viral attachment glycoprotein, play a role in disease pathogenesis. From a therapeutic perspective, it will also be important to determine if soluble forms of the ephrin-B2 and EphB4 extracellular domains, which inhibit henipavirus infection in cell culture, may also be useful as prophylactic agents. Furthermore, various soluble forms of the henipavirus G protein, which binds ephrin-B2 with subnanomolar affinity, may have therapeutic applications to stimulate or inhibit angiogenesis, depending on their ability to activate or block reverse signaling.

Concluding Remarks

Additional roles of Eph receptors and ephrins in adult physiology beyond those discussed in the previous sections have been discovered, and the list continues to grow. For example, hypoxia reportedly stimulates upregulation of ephrin-B2 in bone marrow stromal cells, which in turn activates EphB4 signaling in hematopoietic progenitor cells (Pasquale, 2005). This causes the detachment of the progenitor cells from the stroma and their differentiation into red blood cells, suggesting an Eph-dependent mechanism to maintain oxygen homeostasis in the blood. An involvement of the Eph system in blood clotting has also been demonstrated, where EphA4 and ephrin-B1 expressed in human platelets contribute to the stabilization of the blood clot through an integrin-dependent mechanism (Arvanitis and Davy, 2008). Eph/ephrin-dependent regulation of the permeability of intercellular junctions likely plays a role in glomerular filtration in the kidney. In particular, ephrin-B1 has been recently identified as a potentially important component of the slit diaphragm of podocytes (Hashimoto et al., 2007). Analysis of mutant mice has revealed that EphB2-ephrin-B2 bidirectional signaling controls the ionic homeostasis of the vestibular endolymph fluid in the inner ear and, therefore, has a potential role in vertigo and positional nystagmus (Dravis et al., 2007). Furthermore, given that several Eph receptors and ephrins are expressed in inflammatory cells and upregulated by inflammatory cytokines, the Eph system likely has multiple roles in inflammation (Ivanov and Romanovsky, 2006). EphB-ephrin-B interactions have also been implicated in the development of chronic neuropathic pain following tissue damage (Du et al., 2007). It can be expected that new discoveries clarifying the mechanisms of the known and yet to be discovered Eph physiological activities will keep the spotlight on the Eph field for years to come.

However, several factors could accelerate progress. It is becoming apparent that expression of Eph receptors and ephrins undergoes dynamic spatial and temporal regulation at the transcriptional and posttranscriptional levels, not only during development but also in the adult and probably in diseased tissues. Knowing the relative abundance and cellular localization of Eph receptors and ephrins, and their subcellular localization, is critical for understanding biological function. Therefore, to determine precisely which Eph receptors or ephrins are involved in a particular physiological process, or should be targeted in a particular disease, there is an urgent need for validated and specific antibodies that will enable detailed expression studies. It is also becoming clear that Eph receptors and ephrins can use multiple signaling mechanisms to achieve different effects and that their downstream pathways are often intertwined with other signaling networks. The availability of conditional knockout mice where gene inactivation can be spatially and temporally regulated, and of knockin mice in which a mutated Eph/ephrin replaces the wild-type protein, will be critical for understanding physiological functions and elucidating the in vivo importance of particular downstream signaling pathways. Functional antibodies and chemical genetics approaches also hold great promise for moving the field forward, particularly as more antibodies, peptides, and chemical compounds that can selectively modulate the function of individual Eph receptors and ephrins become available (Himanen et al., 2007; Noren and Pasquale, 2007; Pasquale, 2005). These tools also have the potential to be used for the selective targeting of only a particular Eph/ephrin domain, thus enabling a detailed mechanistic characterization of the multiple activities of these proteins. Systems biology approaches to integrate Eph signaling pathways with other signaling networks will also be helpful. A thorough understanding of Eph-ephrin bidirectional activities will provide new perspectives on physiology, disease pathogenesis, and potential therapies.

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P27-22: Characterization of a Novel 12(S)-HETE Receptor and Role in Prostate Cancer Progression

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Background: 12(S)-hydroxyeicosatetraenoic [12(S)-HETE], a lipoxygenase metabolite of arachidonic acid, enhances metastatic capacity during tumor progression by stimulating several steps of tumor invasion and motility. 12(S) HETE enhances cancer cells to secrete proteases and vascular endothelial growth factor. Treatment of cancer cells with 12(S)-HETE also enhances the expressions of integrins. In endothelial cells, 12(S)-HETE induces the non-destructive retraction of monolayers and promotes tumor cell adhesion. All evidence suggests that 12(S)-HETE is a distinct signaling molecule that evokes a wide variety of cellular responses and influences tumor progression through its receptor. We have cloned a cDNA for the 12(S)-HETE receptor, GPR31, which belongs to the G-protein coupled receptor family. The following experiments are designed to characterize biochemical properties of GPR31 and study the role of GPR31 in tumor cell survival and invasion.

Experiment and Results: (1) We designed 4 constructs for GPR31 shRNA that were cloned into pSilencer5.1-H1 vector. These constructs were transfected into GPR31/CHO cells. Western blot experiments indicated that one of the constructs significantly reduced GPR31 protein expression. (2) We have successfully constructed a pET20b(+)-HIS7Q-GPR31 that was transformed into Rosetta 2 cells. We have obtained pure GPR31 protein using Ni-NTA affinity purification column. (3) We studied the hydrophilicity, antigenicity, and accessibility of GPR31. The peptides, A=163HSFYSRAD GSFSI175, corresponding to second extracellular loop; and B=302GKGQA AEPPDFNPRDSYS319, corresponding to part of the C-terminus of the receptor, are unique to GPR31 and most suitable to antibody production. Therefore, we have synthesized peptides A and B to raise rabbit polyclonal antibodies, B2849 and 250G31, respectively. Using B2849, we examined the expression of GPR31 in control cells (CHO and COS 7), prostate cancer cells (PC3 and DU145), lung cervical cell (A431), and breast cancer cells (11914 and T47). The results indicate that the GPR31 proteins were expressed in cancer cells but not in CHO and COS7 cells. The expression of GPR31 in prostate tumor tissues from two patients also was evaluated using antibody B2849. In one case, the immunoreactivity was rare in normal glands. However, in the neoplastic glands (Gleason grade 3/4), intense brown staining was observed. In another case, strong staining was observed in a Gleason 3/4 tumor area, and weak staining in an area with Gleason grade 3 tumor. This data suggests that GPR31 is present in human prostate cancer tissue.

IMPaCT: This project could potentially have a large impact on the treatment of cancer. GPR31 is expressed specifically on the membrane of cancer cells. As we know, many GPCRs are the targets of pharmacological chemicals. In the future, if we develop a method to knock down its gene expression or find a specific antagonist for this receptor, it could be possible to improve treatment of prostate cancer.

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P27-23: Tumor Suppressor Activity of the EPHB2 Receptor in Prostate Cancer

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Recent work has provided intriguing clues suggesting that EphB2, a member of the large Eph receptor tyrosine kinase family, is a tumor suppressor in prostate cancer. EphB2 mutations were found in clinical prostate cancer samples and not in normal tissue, and cell culture experiments suggested that EphB2 might inhibit the growth of prostate cancer cells. Furthermore, a germline nonsense mutation in the EphB2 gene was recently associated with prostate cancer risk and EphB2 inactivation was reported in human colorectal tumors. However, the mechanisms that may underlie a tumor suppressor role of EphB2 are unknown. Our previous work has shown that activation of EphB receptor signaling pathways by their ligands, called ephrins, can inhibit tumorigenesis in breast cancer cells. We hypothesize that signaling pathways activated by EphB2—and possibly other EphB receptors—also inhibit prostate cancer cell malignancy.

We have compared the expression levels of several Eph receptors and ephrins in non-transformed human BPH-1 prostate epithelial cells and in DU145, PC3, and LNCaP human prostate cancer cell lines. EphB2 protein expression was found to be much lower in the cancer cells than in the BPH-1 cells, consistent with downregulation of EphB2 expression during prostate cancer progression. Furthermore, EphB2 appeared to have a smaller size in the LNCaP cells, suggesting the presence of an alternatively spliced or mutated form. The related EphB3 and EphB4 receptors were expressed at similar levels in the BPH-1 and prostate cancer cells, whereas EphB1 was undetectable, suggesting that the expression of the EphB receptors is regulated differently. Consistent with the hypothesis that activation of EphB receptors may inhibit tumorigenesis, EphB receptor tyrosine phosphorylation was low in the prostate cancer cells, indicating a low level of activation by endogenous ephrin-B ligands. Indeed, we detected substantial levels of ephrin-B1 only in BPH-1 and PC-3 cells, whereas ephrin-B2 and ephrin-B3 were undetectable in all the cell lines. We also found similar EphB2 expression in PIN (prostate intraepithelial neoplasia) tissue from Pten/PB-Cre mice and in normal prostate, indicating that the levels of EphB2 do not change during the initial phases of tumor development in the Pten/PB-Cre mouse model of prostate cancer.

A series of experiments where we transfected EphB2 in DU145 cells suggested that EphB2 inhibits cell growth. To obtain conclusive evidence, we will use an inducible system to compare the same DU145 cell lines with and without EphB2. These inducible cells will also be used for the generation of tumors in nude mice to evaluate whether inactivation of the EphB2 gene indeed accelerates prostate tumor development in vivo. Furthermore, they will be useful to characterize the signaling mechanisms underlying the activities of EphB2.

IMPaCT: Inactivation of the EphB2 gene in prostate and colorectal cancer identifies this receptor as a promising target for therapeutic intervention. Our studies will further characterize the role of EphB2 in prostate cancer and could aid the development of therapies targeting Eph receptors. Furthermore, if we find that EphB2 gene inactivation promotes prostate tumorigenesis in the mouse, future studies will determine if screening clinical specimens for EphB2 expression and gene mutations could be used to discriminate early lesions that are more likely to rapidly progress to malignant prostate cancer.

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P27-24: Canonical WNT Signaling in Prostate Organogenesis

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During embryogenesis, the prostatic buds arise from the rostral end of the urogenital sinus by means of inductive signals from the surrounding mesenchyme. Subsequently, the nascent prostatic epithelia buds undergo ductal outgrowth and branching into the surrounding mesenchyme. We have been investigating the function of the Wnt/β-catenin pathway in these processes of prostate organogenesis, using the mouse as a model system. We have shown that Wnt genes are expressed in the developing urogenital sinus, both in the mesenchyme and epithelium at 15.5 and 16.5 dpc. Wnt1, Wnt2b, Wnt4, Wnt5a, and Wnt5b are spatiotemporally distributed in the mesenchyme at 15.5 dpc and in both the mesenchyme and the epithelium at 16.5 dpc. To define the role of the canonical Wnt pathway in prostate outgrowth we have added exogenous Wnt inhibitors to organ culture of urogenital sinus from Nkx3.1-lacZ embryos, in which lacZ expression is controlled by the endogenous Nkx3.1 promoter. In control cultures, extensive β-galactosidase staining can be visualized in the epithelium of the prostatic buds after 7 days of culture, matching the pattern of Nkx3.1 expression. When cultured in the presence of the Wnt inhibitors, Dkk1, Dkk2, or Dkk3, the intensity of the staining is decreased, and the extent of prostatic budding is reduced. Unexpectedly, however, prostate budding or Nkx3.1 expression is not reduced in the presence of sFRP1. Taken together, we have identified candidate ligands for the canonical Wnt pathway that are likely to play a key role in early prostate organogenesis.

Paradoxes of the EphB4 Receptor in Cancer

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Abstract

Recent findings have started to uncover the intriguing roles of the Eph family of receptor tyrosine kinases in normal epithelial cells and during oncogenic transformation. This review focuses on EphB4, an Eph receptor that has both tumor-suppressing and tumor-promoting activities in breast cancer. Understanding the multifaceted role of EphB4 in tumorigenesis may allow the development of new anticancer therapies. [Cancer Res 2007;67(9):3994–7]

Introduction

The Eph receptors are the largest family of receptor tyrosine kinases. They are divided into an EphA and an EphB class, which respectively bind the glycosylphosphatidylinositol-linked ephrin-A ligands and the transmembrane ephrin-B ligands (1). Although Eph receptors are present in most cell types, thus far, their activities have been best characterized in the developing and adult nervous system and in the developing vascular system. Recent work is beginning to address the functions of the Eph family in the immune system, bone, stem cells, normal epithelial cells, and tumors. Well-known effects of Eph receptor activation by their ephrin ligands involve regulation of cell shape and movement, although modulation of cell survival and proliferation has also been described. Here, we review recent data that begin to shed light on the multiple roles in breast cancer of EphB4, one of the predominant Eph receptors expressed in epithelial cells.

EphB4/Ephrin-B2 Interaction Promotes an Epithelial Phenotype

The EphB4 receptor tyrosine kinase has been detected in the epithelial cells of human breast tissue (2, 3). Furthermore, regulated expression of EphB4 and its preferred ligand ephrin-B2 during development and during the estrous cycle has been proposed to play a role in mammary gland morphogenesis (4). Both EphB4 and ephrin-B2 are transmembrane proteins, and by immunofluorescence microscopy, they seem to be segregated in different membrane microdomains of the MCF-10A nontransformed human mammary epithelial cell line, where they are coexpressed (Fig. 1*A*). This segregated distribution is similar to that previously reported for Eph receptors and ephrins that are coexpressed in neurons (5). Patches of EphB4 immunoreactivity are juxtaposed to patches of ephrin-B2 immunoreactivity in MCF-10A cell-cell junctions (Fig. 1*A* and *B*), consistent with Eph activation occurring at sites of cell-to-cell contact (1). EphB4 is indeed substantially tyrosine phosphorylated in the MCF-10A cells, and treatment with a soluble form of the EphB4 extracellular domain (EphB4 Fc, which blocks the interaction of ephrin-B2 with endogenous EphB receptors) decreases EphB4 tyrosine phosphorylation (6).

Interestingly, EphB4 Fc treatment of MCF-10A cells also inhibits the activity of Abl, a cytoplasmic tyrosine kinase that phosphorylates and inactivates the adaptor protein Crk (6). The Abl/Crk pathway seems to be important for the maintenance of epithelial characteristics in the MCF-10A cells (Fig. 1*B*) because disruption of EphB4/ephrin-B2 complexes with EphB4 Fc or with an EphB4 antagonistic peptide disturbs the organization of cell-cell junctions, suggesting a shift to a more mesenchymal morphology (6). Consistent with these results, Crk adaptor activity has been shown to induce epithelial-to-mesenchymal transition (7). EphB4 Fc treatment also promotes other aspects of cell transformation, including increased cell growth in two-dimensional cultures and in soft agar (6).

Given that activation of receptors of the EphA class has also been reported to promote epithelial characteristics in cells of the mammary gland, kidney, and somites (1, 8), it will be important to determine whether other Eph receptors also regulate the Abl/Crk pathway. The finding that EphB4 Fc, which should not interfere with EphA signaling, greatly inhibits Crk phosphorylation suggests that EphA receptors may signal predominantly through other pathways in mammary epithelial cells. For example, ligand-stimulated EphA2 inhibits the Ras/mitogen-activated protein kinase pathway (9, 10). It should be noted that the ligand ephrin-B2 also has the ability to generate signals through its cytoplasmic domain, which are known as reverse signals (1). These signals can be stimulated by endogenous EphB4 or by EphB4 Fc (1, 11) and may contribute to maintain an epithelial phenotype (12), but their identity in epithelial cells is unknown (Fig. 1*B*).

EphB4 Signaling Inhibits Breast Cancer Cell Tumorigenicity

Up-regulation of EphB4 expression has been found in mouse mammary tumor models and in more than half of the human breast cancer specimens examined (2–4, 13). EphB4 is also widely expressed in human breast cancer cell lines (6, 13). Increased EphB4 expression may be a common occurrence during epithelial cell transformation because it has been reported in many types of cancer (refs. 6, 13–15 and references therein). Several signaling pathways involved in tumorigenesis can indeed promote EphB4 expression, including the Janus-activated kinase/signal transducer and activator of transcription and phosphatidylinositol 3-kinase/Akt pathways downstream of ErbB family receptors and the Wnt/β-catenin/Tcf4 pathway (13, 14). Estrogen has also been shown to drive EphB4 expression in the mouse mammary gland (4). In addition, EphB4 is located on chromosome 7 in a region (7q22.1) that is frequently amplified in breast cancer, and *EphB4* gene amplification has indeed been detected in several breast cancer cell lines (13).

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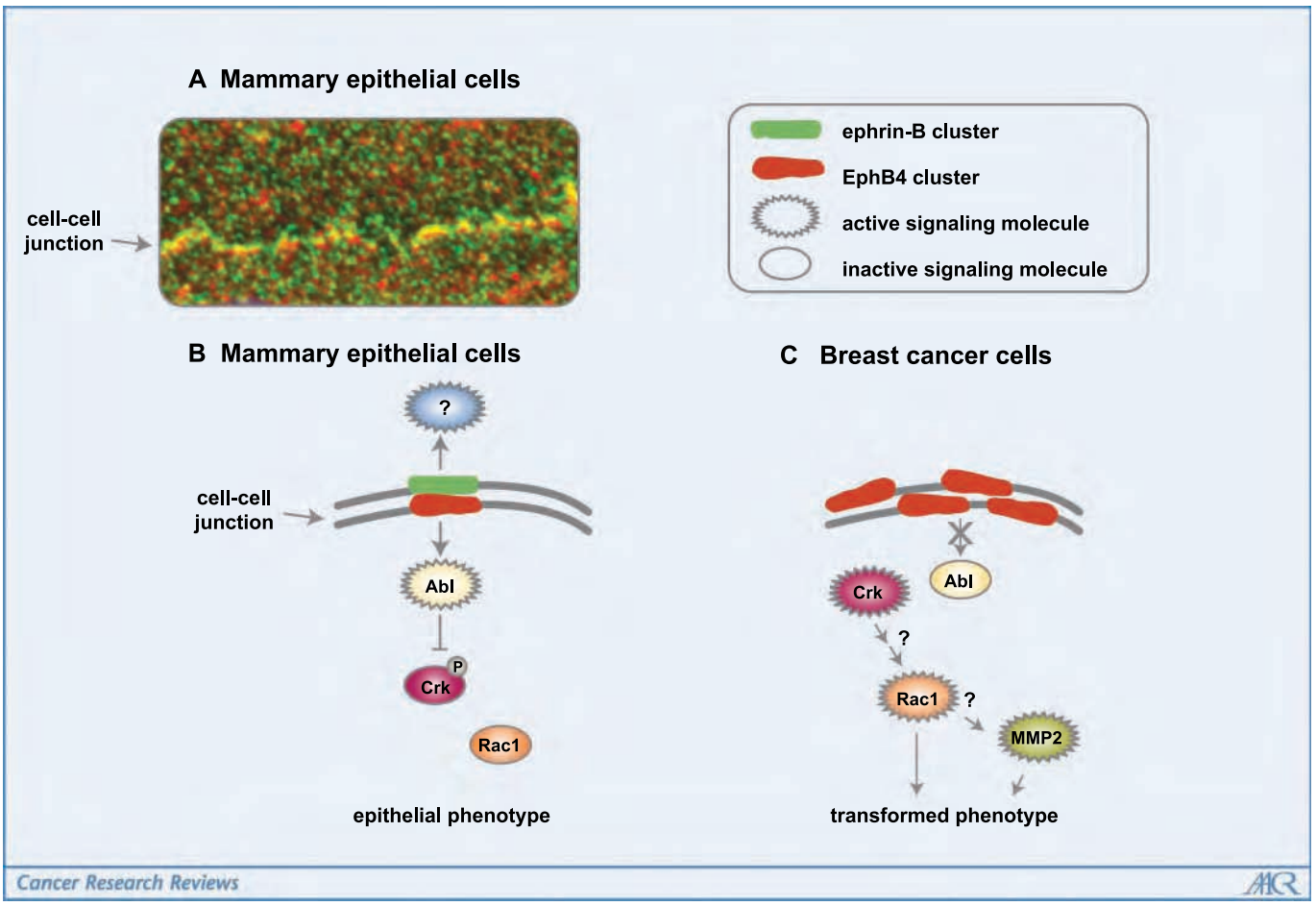

Figure 1. Signaling by the EphB4 receptor to the tyrosine kinase Abl and the adaptor Crk is high in mammary epithelial cells and low in breast cancer cells. *A,* MCF-10A nontransformed mammary epithelial cells were stained with EphB4 antibodies (*red*) and ephrin-B antibodies (*green*). Ephrin-B2 is the predominant B class ephrin in these cells. The arrow marks the junction between two cells. Note that EphB4 and ephrin-B2 seem to be localized in different membrane microdomains throughout the cell and are juxtaposed at the cell-cell junction. *B,* schematic representation of EphB4/ephrin-B2 clusters in MCF-10A cell-cell junctions. The signaling pathways initiated by EphB4 and ephrin-B2 in epithelial cells lead to Abl activation and inactivation of Crk adaptor function through phosphorylation by Abl. *C,* ephrin-B2 expression is low in breast cancer cells, which causes a decrease in EphB4 and Abl activity, resulting in increased Crk adaptor function and MMP2 expression, presumably through activation of the small GTPase Rac1. Cell membrane (*gray*). Abl represents both Abl and the related kinase Arg.

Surprisingly, despite the substantial levels of EphB4 expression, EphB4 tyrosine phosphorylation is much lower in breast cancer cell lines compared with nontransformed MCF-10A epithelial cells (6). Furthermore, in breast cancer cells, Abl kinase activity is lower, and Crk is less tyrosine phosphorylated and therefore better able to function as an adaptor protein (Fig. 1*C*). The silencing of EphB4 signaling in breast cancer cells is consistent with the low expression of ephrin-B2 in these cells. Loss of ephrin-B2 has also been reported in mouse mammary tumor models (4), and ephrin-B2 is down-regulated by the Wnt/β-catenin/Tcf4 pathway in colorectal cancer cells (14). Furthermore, allelic losses have been described in various cancers for the chromosome 13q33 region, where the *ephrin-B2* gene is located (16).

The low EphB4 tyrosine phosphorylation in mammary tumor cells suggested that ligand-stimulated signaling through the EphB4 cytoplasmic domain may be detrimental to tumor development. Indeed, EphB4 inhibits breast cancer cell tumorigenicity both *in vitro* and *in vivo* when its tyrosine kinase activity is stimulated by a soluble form of the ligand, ephrin-B2 Fc (6). Treatment of several breast cancer cell lines with ephrin-B2 Fc inhibited proliferation and increased apoptosis. Furthermore, ephrin-B2 Fc inhibits breast cancer cell motility and invasion, concomitant with decreased expression of the matrix metalloprotease MMP2. Increased activity not only of Abl but also the related Arg kinase contributes to Crk phosphorylation in breast cancer cells activated with ephrin-B2 Fc. A series of experiments using Abl and Crk mutants, RNA interference, and the Abl/Arg kinase inhibitor Gleevec showed that restoration of the EphB4/Abl/Crk pathway is responsible for the anti-oncogenic effects of ephrin-B2 Fc in cell culture and *in vivo* in a breast cancer mouse xenograft model (6).

Oncogenic forms of Abl, such as BCR-Abl, have dysregulated kinase activity and aberrant subcellular localization and thus mediate inappropriate signaling pathways (17). In contrast, the activities of cellular Abl downstream of EphB4 in breast cancer cells are consistent with previous reports that Abl can inhibit cell motility and invasion as well as promote apoptosis through inhibitory phosphorylation of Crk (18, 19). A likely mediator of the effects of Crk in breast cancer cells is the Rac1 GTPase (refs. 7, 18; Fig. 1*C*). Crk can promote Rac1 activation by forming signaling complexes with the scaffolding protein Cas and the Rac1 exchange factor DOCK180, and ephrin-B2 Fc treatment does indeed disrupt Crk/Cas complexes in breast cancer cells (6, 18). Additional

pathways that are operational in epithelial cells involve complexes of Crk with the scaffolding protein paxillin, the ARF-GAP GIT2, and the Rac1 exchange factor β-PIX or with the scaffolding protein Gab1. Thus, a decrease in EphB4 activation and the resulting up-regulation of Crk downstream signaling pathways leading to Rac1 activation may contribute to breast cancer initiation and progression (Fig. 1*C*). In contrast, the Rap1 GTPase, which is also activated downstream of Crk in complex with Cas, does not seem to play a critical role (7, 19).

Increasing evidence shows that oncogenic signaling pathways also up-regulate the expression of other Eph receptors, including EphA2, EphB2, and EphB3, in cancer cells (9, 10, 14, 15). However, like EphB4, these receptors have been shown to inhibit tumorigenesis in at least some cancer types. For example, EphA2 inhibits cell proliferation and promotes epithelial cell morphology when activated by the ligand ephrin-A1 (8–10). In the case of EphA2 in skin tumors and of several EphB receptors in colorectal tumors, it has also been proposed that interactions between the Eph receptor–positive tumor cells and the surrounding ephrin-positive normal epithelial cells compartmentalizes the tumor and prevents tumor cells from expanding and infiltrating the normal tissue (10, 14). Interestingly, there is evidence to suggest that the tumors overexpressing Eph receptors may be the less malignant ones, and that Eph receptor expression is lost in the more advanced stages (e.g., through promoter methylation or gene mutations; refs. 2, 9, 10, 14, 15, 20). One study indicates that this may also be the case for EphB4 in breast cancer (2). Thus, cancer cells may elude the tumor suppressor activities of Eph receptors by down-regulating ephrin or Eph receptor expression. Additionally, mutations that impair Eph receptor signaling ability or up-regulation of tyrosine phosphatases that dephosphorylate Eph receptors may also promote tumorigenesis (20, 21). Taken together, the available information suggests that Eph-ephrin interactions and signaling in normal epithelial tissues help maintain tissue homeostasis, and that their disruption may be a factor in the development and progression of cancer.

Tumor-Promoting Effects of EphB4

In addition to its tumor suppressor activity in breast cancer, the EphB4 receptor can also promote tumorigenesis through different mechanisms. The EphB4 extracellular domain can induce angiogenic responses by stimulating ephrin-B2 reverse signaling in cultured endothelial cells (11). In agreement with this, EphB4 expressed on the surface of breast cancer cells has been shown to promote angiogenesis in tumor xenografts by activating ephrin-B2 reverse signaling in the vasculature, thus increasing tumor growth (11). Tumor angiogenesis may also have contributed to the increased tumor growth observed in a mouse mammary tumor model with transgenic overexpression of EphB4 in epithelial cells of the mammary gland (4). EphB4 expressed in tumor endothelial cells has also been shown to predominantly function by stimulating reverse signaling through endothelial ephrin-B2 (13, 22).

Additional tumor-promoting effects of EphB4 that are independent of angiogenesis have also been identified by down-regulating EphB4 using RNA interference and antisense oligonucleotide approaches. EphB4 knockdown was found to reduce survival, proliferation, migration, and invasion of breast cancer cells and many other types of cancer cells (ref. 13 and other articles by the same group). In these cells, EphB4 was generally found to be poorly tyrosine phosphorylated, suggesting that the tumor-promoting ability of this receptor is independent of ligand-mediated kinase activation (1). Similarly, the EphA2 receptor overexpressed in MCF-10A cells is poorly tyrosine phosphorylated and promotes oncogenic transformation, an effect that is reversed by treatment of the cells with the ligand ephrin-A1 Fc (8). In addition, the low molecular weight tyrosine phosphatase promotes transformation of MCF-10A cells by dephosphorylating EphA2 (21). Whether signaling pathways that are independent of ephrin-mediated Eph receptor phosphorylation and crosstalk with oncogenic or apoptotic signaling pathways may explain the tumor-promoting effects of Eph receptors remains to be determined.

Further adding to the complexity of EphB4 function in cancer cells, it has also been reported that in some types of cancers, such as melanoma, ephrin-B2–dependent EphB4 signaling enhances the migratory and invasive ability of the cells (23). These effects require EphB4 signaling and activation of the RhoA GTPase. In addition, signaling by another Eph receptor (EphB2) has been shown to promote the invasive ability of human glioma cells through phosphorylation of the R-Ras GTPase (24). Interestingly, however, the EphB2/R-Ras pathway inhibits glioma cell proliferation. Hence, the cellular context also seems to play an important role in determining the tumor-promoting or tumor-suppressing effects of Eph receptors in cancer.

EphB4 as a Breast Cancer Target

The widespread expression of EphB4 and other Eph receptors in tumors has stimulated interest in exploring these receptors as targets for the development of new cancer therapies. Given the different activities of EphB4 in breast cancer cells, the most effective design for EphB4-based breast cancer treatments should be to inhibit EphB4 binding to endothelial ephrin-B2 while at the same time promoting EphB4 downstream signaling. The soluble form of the ligand (ephrin-B2 Fc) can promote EphB4 activation and, at higher concentrations, inhibit endogenous EphB4/ephrin-B2 interaction. Ephrin-B2 Fc administered systemically at low concentrations has already been shown to inhibit the growth of breast cancer xenografts in nude mice by activating the EphB4/Abl/Crk pathway (6). It will be interesting to examine whether higher doses of ephrin-B2 Fc may be even more effective by also inhibiting tumor angiogenesis. However, ephrin-B2 Fc can bind all the EphB receptors and also EphA4 (1), increasing the potential for unwanted effects. More selective reagents, such as EphB4-activating antibodies, might be more suitable to specifically inhibit the interaction of EphB4 with endothelial ephrin-B2 and also activate EphB4 downstream anti-oncogenic signaling pathways.

A soluble monomeric form of the EphB4 extracellular domain, which can inhibit both EphB4 signaling and ephrin-B2 reverse signaling, has also been useful for decreasing tumor growth in several mouse tumor xenograft models, including a breast cancer model (25, 26). This suggests that peptides and small molecules that inhibit EphB4/ephrin-B2 interaction (27), which may have more desirable therapeutic properties and cost-effectiveness than the large EphB4 extracellular domain, represent promising agents to inhibit tumor angiogenesis. Furthermore, they could inhibit EphB4 tumorigenic signaling pathways in certain cancers such as melanoma (23). Down-regulation of EphB4 expression with antisense oligonucleotides has also been an effective strategy to inhibit the growth of breast and other types of tumor xenografts expressing high levels of EphB4 (13). This approach can both inhibit EphB4-dependent angiogenesis and counteract possible kinase-independent EphB4 signaling pathways.

Inhibition of EphB4 kinase activity using ATP analogues will be useful against those tumors, such as melanoma, where EphB4 kinase activity promotes tumorigenesis. Kinase inhibitors may instead be ineffective or even detrimental for the treatment of breast cancer and other types of cancer where EphB4 signaling suppresses tumorigenesis. This is also the case for the Abl kinase inhibitor Gleevec, although Gleevec has proven to be a very effective therapy for targeting oncogenic BCR-Abl in chronic myelogenous leukemia patients (17). Tumor xenograft studies show that Gleevec can counteract the anti-oncogenic effects of EphB4 agonists in breast cancer and should therefore not be used in combination with them (6). On the other hand, chemotherapeutic agents that target ErbB receptors or taxol may enhance the effects of EphB4-targeted therapies (13, 28). Finally, antibodies, peptides, and small molecules that bind to EphB4 but lack intrinsic biological activity could be coupled to toxic substances to selectively kill tumor cells that overexpress the receptor.

Our understanding of the complex roles of EphB4 and other Eph receptors in cancer is still evolving, and more information is needed to resolve the many confusing and controversial issues. Future research will determine whether EphB4-based therapeutic strategies can be effective for the treatment of cancers that overexpress EphB4 and in which types of cancer different therapeutic approaches may be most appropriate. It will also be important to examine the effects of EphB4-targeting agents on normal epithelial cells *in vivo*. New insights into Eph signaling pathways in normal and tumor cells will be important not only for the development of new cancer therapies but also for the optimal use of existing therapies.

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EPH RECEPTORS AND EPHRINS

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The Eph receptors are a large family of receptor tyrosine kinases expressed in many developing and adult tissues, including blood and lymphatic vessels. Interaction of the Eph receptors with their ligands (the ephrins), which are membrane-associated, mediates contact-dependent cell-to-cell communication. Expression studies indicate that in the embryonic vascular system, Eph receptor-ephrin interactions can occur at contact sites between adjacent endothelial cells in the same vessel, between arterial and venous endothelial cells, and between endothelial cells and surrounding cells. The EphB4 receptor and its preferred ligand, ephrin-B2, have a striking distribution in embryonic venous and arterial endothelial cells, respectively, and are essential for the development of functional blood vessels. Several other Eph receptors and ephrins also play critical roles in the angiogenic sprouting and remodeling of embryonic blood vessels and in pathological forms of angiogenesis, such as tumor angiogenesis. Crosstalk with other families of angiogenic molecules likely contributes to these functions. In the lymphatic system, Eph receptors and ephrins are also required for vessel morphogenesis. Because of their important functions in the vasculature, Eph receptors and ephrins are emerging as new targets for therapies to promote or inhibit angiogenesis.

1. Introduction

The Eph family comprises ten EphA receptors (EphA1-EphA10) and six EphB receptors (EphB1-EphB6) in vertebrates (1). EphA9 and EphB5, however, were identified in chicken and do not appear to be present in mammals. The Eph receptors influence the behavior of many cell types, unlike other families of receptor tyrosine kinases such as the vascular endothelial growth factor (VEGF) receptor family and the Tie family of angiopoietin receptors, which function more selectively in blood vessels (Chapters 15 and 16). The first Eph receptor was identified from a human erythropoietin-producing hepatocellular carcinoma cell line in 1987 and named Eph (and later renamed EphA1) (2,3). Identification of additional Eph receptors and characterization of their expression patterns suggested important roles in the formation of connections between neurons during the wiring of the developing nervous system as well as in the organization of epithelial structures and the vasculature. Functional characterization of the Eph receptors was greatly facilitated by the identification of the ligands that stimulate the signaling activity of these receptors. The first ligand for an Eph receptor to be identified was B61, later renamed ephrin-A1 (Eph receptor interacting protein A1) (4).

Studies with ephrin-A1, and its receptor EphA2, were the first to reveal a role for Eph receptors and ephrins in angiogenesis (5). These seminal findings were followed by numerous genetic studies in the mouse, which have uncovered the involvement of several Eph receptors and ephrins in the normal development of blood and lymphatic vessels as well as in pathological

forms of angiogenesis. The complexities in the expression patterns and signaling mechanisms of Eph receptors and ephrins have so far precluded a detailed understanding of the molecular mechanisms used by these molecules to influence the vasculature in vivo. However, in vitro studies with vascular cells and in vivo angiogenesis assays have shown that ephrin-Eph receptor signaling can regulate many properties of vascular cells – including their shape, adhesion, migration, and proliferation – and promote the assembly of capillary-like structures and capillary sprouting.

1.1. Eph Receptor Domain Structure

The Eph receptors have an extracellular region that comprises the ephrin-binding domain at the amino terminus, a cysteine-rich region containing an epidermal growth factor (EGF)-like repeat, and two fibronectin type III repeats (Fig. 1). A single membrane-spanning segment connects the extracellular portion of the Eph receptors to the cytoplasmic portion, which comprises a juxtamembrane segment, the tyrosine kinase domain and a sterile alpha motif (SAM) near the carboxy terminus. Most Eph receptors have a PDZ domain-binding site at their extreme carboxy terminus. Following ephrin binding, the Eph receptors dimerize and further cluster, which leads to phosphorylation of many of their cytoplasmic tyrosines (1). Several of these phosphorylation sites release the kinase domain from inhibitory interaction with the juxtamembrane segment, thereby promoting kinase activity. Furthermore, the tyrosine phosphorylated motifs recruit cytoplasmic signaling proteins containing SH2 domains, leading to activation of downstream signaling pathways (1). Two Eph receptors, EphA10 and EphB6, lack residues required for kinase activity and likely can be phosphorylated only as a result of crosstalk with other Eph receptors or with other signaling molecules. Alternatively spliced forms of the Eph receptors differ from the prototypical Eph receptor described above in their domain structure and, therefore, their function. For example, variant forms of some Eph receptors lack the kinase domain and may be primarily involved in cell adhesion rather than signaling (6).

1.2. The Ephrin Domain Structure

The ephrins are membrane-bound molecules, which is unusual for ligands of receptor tyrosine kinases (Fig. 1). The six vertebrate ephrin-A molecules (ephrin-A1 to ephrin-A6) are associated with the cell surface through a glycosylphosphatidylinositol (GPI) linkage and the three ephrin-B molecules (ephrin-B1 to ephrin-B3) are transmembrane proteins that also contain a cytoplasmic segment. Ephrin-A6, however, was identified in the chicken and does not appear to be present in mammals. The extracellular region of both A and B class ephrins is almost entirely occupied by the receptor-binding domain, which is connected to the plasma membrane through a linker region. The cytoplasmic portion of the ephrin-B ligands is highly conserved and contains a PDZ

domain-binding site at the extreme carboxy terminus. Interaction with Eph receptors can stimulate phosphorylation of tyrosine residues in the ephrin-B cytoplasmic domain through the activity of Src family kinases (1,7). These phosphorylation events affect the conformation of the cytoplasmic domain (8) as well as recruit signaling proteins (9).

1.3. Eph-Ephrin Bidirectional Signaling at Sites of Cell-to-Cell Contact

The EphA receptors bind preferentially ephrins of the A class and the EphB receptors bind preferentially ephrins of the B class, and interactions between receptors and ephrins of the same class are very promiscuous (10). In addition, EphA4 and EphB2 can bind ligands of the other class. An important exception in the vascular system is the EphB4 receptor, which binds with high affinity only to ephrin-B2. Because both Eph receptors and ephrins are present on the cell surface, their interactions are restricted to sites of cell-to-cell contact (1). In the vascular system, Eph receptor-ephrin interactions may occur at contact sites between endothelial cells in the same vessel, between venous and arterial endothelial cells and between endothelial and mesenchymal vascular support cells (Fig. 2). Since signals can be generated by both the Eph receptors and the ephrins, bidirectional signals can emanate from sites of cell-to-cell contact and affect both cells. These signals can lead to repulsive effects involving retraction of the cellular processes that were initially engaged in cell-to-cell contact or to attractive effects, such as increased cell-cell and cell-substrate adhesion and forward movement (1). The outcome of the signals may depend on the degree of Eph receptor activation and clustering, and possibly other as yet unidentified factors. For example, EphA2 signaling mediates positive chemotactic signals and pro-angiogenic effects in endothelial cells, whereas it mediates repulsive effects and apoptosis in cancer cells (5,11,12).

2. Effects on Vascular Cell Behavior and Signaling Pathways

Eph receptor forward signaling and ephrin reverse signaling can dramatically influence the behavior of endothelial cells and vascular smooth muscle cells both in vitro and in angiogenesis assays in vivo. The signaling pathways regulated by Eph receptors and ephrins in vascular cells are beginning to be elucidated through in vitro studies, but the importance of these pathways for physiological angiogenesis in vivo remains to be verified. Ephrin extracellular domains fused to the Fc portion of human IgG_1 have been extensively used to activate Eph receptor signaling pathways in angiogenesis assays because they are soluble and dimeric, and can be multimerized by anti-Fc antibodies. Thus, they can be used to induce the Eph receptor dimerization and further clustering that are important for proper signaling (1,13). Similarly, Eph receptor ectodomains fused to Fc have been used to stimulate ephrin reverse signaling. Eph receptor and ephrin Fc

fusion proteins, however, can also function as inhibitors because they disrupt endogenous Eph receptor-ephrin interactions. For example, EphA receptor Fc fusion proteins have been used to inhibit EphA forward signaling, which has established the importance of EphA receptors in postnatal angiogenesis (14,15) (section 5).

2.1. Ephrin-A1 and EphA2

Ephrin-A1 and EphA2 are the main ephrin and Eph receptor of the A class that have thus far been implicated in endothelial cell function (Fig. 2A). In a widely used in vitro angiogenesis model, endothelial cells plated onto reconstituted basement membrane proteins (Matrigel) respond by forming capillary tube-like structures. Interestingly, one of the consequences of plating human umbilical vein endothelial (HUVE) cells on Matrigel is the upregulation of ephrin-A1 (16). The effect of ephrin-A1 in capillary morphogenesis on Matrigel was confirmed by showing that exogenously added ephrin-A1 Fc promotes the assembly of capillary structures in HUVE cells and mouse pulmonary microvascular endothelial cells, both of which express high levels of the EphA2 receptor (5,17). Furthermore, reducing expression of the transcription factor Homeobox B3 (HoxB3) with antisense oligonucleotides decreases ephrin-A1 expression and impairs capillary morphogenesis in dermal microvascular endothelial cells (18). Given that treatment with ephrin-A1 Fc restores capillary-like tube formation in the HoxB3-deficient cells, these data suggest that the HoxB3-dependent expression of ephrin-A1 is important for endothelial capillary morphogenesis driven by extracellular matrix proteins. Not all endothelial cells may respond to ephrin-A1 Fc, however. Human renal microvascular endothelial cells, for example, reportedly do not form capillary-like tubes in response to ephrin-A1 Fc (17). The reason for this lack of responsiveness to ephrin-A1 Fc remains mysterious, since these cells express EphA2 and can form capillary-like tubes when treated with ephrin-B1 Fc (section 2.3).

Consistent with a role as an angiogenic factor, ephrin-A1 Fc also promotes endothelial cell migration. Ephrin-A1 Fc acts as a chemoattractant for bovine adrenal capillary endothelial cells and microvascular endothelial cells in transwell migration assays and promotes the movement of cells into a “wound” devoid of cells in a confluent endothelial cell monolayer (5,14,19,20). Ephrin-A1 Fc also induces endothelial cell sprouting in an in vitro capillary sprouting assay (19). In this assay, microvascular endothelial cells are cultured on collagen-coated beads embedded in fibrin gels and form capillary sprouts that extend out from the beads into the fibrin matrix.

Additional studies have shown that ephrin-A1 Fc also promotes the formation of blood vessels in a variety of in vivo angiogenesis assays. These assays include: (i) corneal neovascularization assays, where hydron pellets impregnated with ephrin-A1 Fc induce the formation of blood vessels when implanted in a micropocket in the normally avascular rodent

cornea (5,19); (ii) Matrigel assays, where Matrigel injected under the mouse skin forms plugs that promote the assembly of endothelial cells into blood vessels when supplemented with ephrin-A1 Fc; and (iii) assays in which surgical sponges impregnated with ephrin-A1 Fc and implanted in the dorsal flank of mice attract an increased number of host blood vessels compared to control sponges (20).

Ephrin-A1 appears to have similar pro-angiogenic effects when it is endogenously expressed in endothelial cells and as an exogenous Fc fusion protein. Hence, the angiogenic effects of ephrin-A1 can be mainly attributed to its stimulation of EphA receptor forward signaling because ephrin-A1 Fc lacks the ability to mediate reverse signals. Indeed, mutants of the EphA2 receptor that inhibit EphA receptor forward signaling in a dominant negative manner block the in vitro angiogenic effects of ephrin-A1 Fc, while a constitutively active EphA2 mutant enhances angiogenic responses (20,21). Furthermore, an EphA antagonist such as EphA2 Fc strongly inhibits capillary formation in an in vitro rat aortic ring explant assay and in an in vivo Matrigel assay (15). Since EphA2 Fc can also activate ephrin-A1 reverse signaling (section 2), this experiment corroborates the idea that ephrin-A1 reverse signaling may not promote angiogenesis.

The requirement for EphA2 signaling in endothelial cell migration and vascular assembly has been confirmed by the impaired angiogenic responses to ephrin-A1 Fc in microvascular endothelial cells isolated from EphA2 knockout mice as well as in endothelial cells in which EphA2 expression was downregulated with antisense oligonucleotides (19,20). A signaling pathway involving phosphatidylinositol (PI) 3 kinase and the Rho family GTPase, Rac1, has been implicated in the effects of EphA2 on microvascular endothelial cell migration in vitro (20,22). Although EphA2 is not expressed in the embryonic vasculature, this receptor has been confirmed as a key player in postnatal angiogenesis in vivo because EphA2 knockout mice exhibit a diminished angiogenic response to surgical sponges impregnated with ephrin-A1 Fc (20). Furthermore, microvascular endothelial cells from EphA2 knockout mice fail to elongate and assemble into capillaries in Matrigel plugs implanted into wild-type recipient mice. The EphA2-deficient cells also have impaired survival in the Matrigel plugs, but appear to proliferate normally, which is consistent with the lack of in vitro effects of ephrin-A1 Fc on endothelial cell proliferation (19).

2.2. Ephrin-A1 and EphA4

Endothelial ephrin-A1 likely coordinates different aspects of angiogenesis by activating EphA receptors not only in endothelial cells, but also in the surrounding vascular smooth muscle cells (Fig. 2A). In cultured smooth muscle cells, ephrin-A1 Fc causes a repulsive response involving increased assembly and contractility of actin stress fibers and decreased cell substrate adhesion

(23). These effects occur through activation of the EphA4 receptor, which is highly expressed in vascular smooth muscle cells. EphA4 in turn activates an exchange factor of the Ephexin family with selective expression in vascular smooth muscle cells, Vsm-RhoGEF, which increases RhoA activity (23). Ephrin-A1 Fc treatment also impairs smooth muscle cell spreading on extracellular matrix proteins by inactivating another Rho family GTPase, Rac1, and its downstream effector p21-activated kinase 1 (Pak1) (24). These data support the idea that endothelial ephrin-A1 coordinates the angiogenic responses of both endothelial and vascular support cells by eliciting different responses in these two cell types. By inhibiting the spreading and promoting the contractility of smooth muscle cells, ephrin-A1 may destabilize their interaction with endothelial cells, allowing endothelial cell migration and vascular assembly into new capillary sprouts. These effects all seem to depend on EphA forward signaling. Whether reverse signals mediated by ephrin-A1 may have additional roles in angiogenesis remains to be determined.

2.3. Ephrin-B and EphB

2.3.1. EphB forward signaling

EphB receptor forward signaling also affects the properties of endothelial cells. Ephrin-B1 Fc stimulates the formation of capillary structures in human renal microvascular endothelial cells, which express the EphB1 and EphB2 receptors (13,17). Ephrin-B1 Fc and ephrin-B2 Fc also induce capillary sprouting in adrenal cortex-derived microvascular endothelial cells with a potency comparable to that of angiopoietin-1 and VEGF (25). However, there is some selectivity with regard to endothelial cell type because ephrin-B1 Fc does not induce capillary-like tubes in HUVE cells (17), which express the EphB1, EphB2, EphB3, and EphB4 receptors (26). This suggests that EphB forward signaling is not sufficient to mediate capillary assembly in HUVE cells, although it should be noted that ephrin-B1 does not efficiently activate EphB4. Nevertheless, ephrin-B1 Fc reportedly promotes HUVE cell proliferation and migration (27). Ephrin-B2 Fc, on the other hand, promotes the proliferation and migration of mesenteric microvascular endothelial cells (28).

In the renal microvascular endothelial cells, ephrin-B1 Fc promotes capillary-like assembly only when clustered with anti-Fc antibodies, whereas dimeric ephrin-B1 Fc is ineffective, even though in both cases EphB receptor tyrosine phosphorylation is similarly induced (13). In addition, tetrameric ephrin-B1 Fc promotes cell-substrate adhesion and migration but the dimeric ephrin does not (13,29). EphB-dependent stimulation of endothelial cell attachment is mediated by the $\alpha_v\beta_3$ integrin and depends on the density of ephrin-B1 Fc immobilized on the surface on which the cells grow (and therefore the degree of EphB receptor clustering), with highest adhesion at intermediate levels of clustering (29). Sprouting

angiogenesis of adrenal cortex microvascular endothelial cells induced by ephrin-B2 Fc (but not ephrin-B1 Fc) also requires clustering of the ephrin (25). In addition, pellets containing ephrin-B2 Fc induce the formation of blood vessels in corneal micropocket assays, although more weakly than pellets containing VEGF (30). Interestingly, in this assay ephrin-B2 Fc preferentially promotes venous neovascularization, as judged by the few ephrin-B2 positive vessels induced and by the upregulation of EphB4 mRNA.

These results suggest that there are differences in the signaling pathways activated by different EphB receptor oligomeric forms. Indeed, low-molecular-weight phosphotyrosine phosphatase (LMW-PTP) is only recruited to EphB1 and EphB2 when these receptors are activated by clustered ephrin-B1 Fc, and mutants of EphB1 that cannot bind LMW-PTP phosphatase fail to promote microvascular endothelial cell attachment and capillary-like assembly (13). Another signaling cascade that has been implicated in promoting cell adhesion downstream of EphB1 and EphB2 involves the adaptor Nck, the Nck-interacting serine/threonine kinase (NIK), and activation of the c-Jun N-terminal kinase (JNK) (31). This Nck-NIK-JNK pathway remains to be verified in endothelial cells, however. EphB1-mediated stimulation of renal microvascular endothelial cell migration also requires Nck, in this case to couple EphB1 to paxillin, which is phosphorylated by activated Src and promotes cell migration (32). EphB1-dependent cell migration also requires phosphorylation of the adaptor Shc by Src, which promotes binding of Shc to another adaptor, Grb2, leading to activation of the Ras-mitogen-activated protein (MAP) kinase pathway (33). The Crk adaptor protein has also been implicated in endothelial cell spreading and migration downstream of EphB1. When human aortic endothelial cells are stimulated with ephrin-B1 Fc, Crk promotes membrane ruffling through the Rho family GTPase Rac1 and focal complex assembly and cell spreading through the Ras family GTPase Rap1 (34). Other studies have shown the involvement of the phosphatidylinositol-3 (PI-3) kinase-Akt pathway in EphB-dependent endothelial cell migration in vitro as well as in corneal and Matrigel neovascularization in vivo (28,35).

In contrast to the attractive effects of EphB receptors described above, other reports have shown that ephrin-B2 Fc stimulation of EphB forward signaling mediates repulsive effects in HUVE cells, in the brain-derived bEnd3 capillary endothelial cell line, and in FACS-sorted EphB4-positive mouse embryonic endothelial cells (26,36,37). For example, stimulation of EphB4 forward signaling prevents endothelial cell attachment and spreading on immobilized ephrin-B2 Fc as well as inhibits proliferation, capillary-like assembly, and sprouting angiogenesis. Ephrin-B2 Fc also inhibits the migration and proliferation of HUVE cells stimulated with VEGF or angiopoietin-1 (section 2.4), at least in part through the recruitment of the Ras GTPase-activating protein p120RasGAP to activated EphB receptors and subsequent suppression of the Ras-MAP kinase pathway (26). EphB4 forward signaling also causes anti-

adhesive effects that disrupt the integrity of the endothelial monolayer in spheroids of co-cultured endothelial and smooth muscle cells as well as in umbilical cord explants (37). The repertoire of EphB receptors activated, the presence of co-expressed ephrin-B ligands, or other as yet unknown factors, may influence the type of response mediated by an activated EphB receptor.

2.3.2. Ephrin-B reverse signaling

Ephrin-B reverse signaling causes pro-angiogenic effects in a variety of endothelial cell types. In HUVE and bEnd3 cells treated with EphB4 Fc, ephrin-B2 reverse signals enhance endothelial cell attachment and spreading on immobilized EphB4 Fc and increase cell survival, migration, sprouting angiogenesis in a collagen gel, and capillary-like assembly on Matrigel (36-38). EphB3 Fc and EphB4 Fc also induce angiogenic sprouting in cultured adrenal cortex-derived endothelial cells (39) and EphB1 Fc promotes attachment and migration of renal microvascular endothelial cells in vitro and blood vessel formation in vivo in a mouse corneal neovascularization assay (40). A mixture of EphB1 Fc and EphB3 Fc has also been shown to increase the number and length of microvessel sprouts in a rat aortic ring angiogenesis assay (15). While EphB1, EphB2, and EphB3 Fc fusion proteins could activate reverse signals through any of the three ephrin-B ligands, EphB4 Fc more selectively activates only ephrin-B2.

Ephrin-B2 reverse signaling may be responsible for some of the pro-angiogenic effects reported for the EphB4 receptor in HUVE cells. EphB4 expression is upregulated by the transcription factor HoxA9 and contributes to the in vitro angiogenic effects of HoxA9 in HUVE cells, because reducing EphB4 expression with siRNA or antisense oligonucleotides impairs HoxA9-dependent HUVE cell migration and capillary-like assembly (41). Consistent with the idea that EphB4 acts by stimulating ephrin-B2 reverse signaling, transfection of a dominant negative form of EphB4 that should block endogenous EphB receptor forward signaling while still mediating reverse signaling fails to inhibit HUVE cell capillary-like assembly on Matrigel (21).

Src family kinases, which become activated upon ephrin-B stimulation and phosphorylate the ephrin cytoplasmic domain, have been implicated in the angiogenic effects mediated by ephrin-B reverse signaling (7,42). Ephrin-B reverse signals also activate integrins to promote renal microvascular endothelial cell attachment and migration (40). These angiogenic effects may involve an as yet unknown protein that binds to the ephrin-B carboxy terminus and mediates activation of JNK, although this signaling pathway remains to be verified in endothelial cells. In addition, the PI3 kinase-Akt and MAP kinase signaling pathways have been implicated in the angiogenic effects of ephrin-B2 reverse signaling in retinal endothelial cells (42). It is not known whether different ephrin-B molecules activate distinct angiogenic pathways.

Based on the in vitro attractive effects of ephrin-B2 and the repulsive effects of EphB4 in endothelial cells, a model has been proposed where ephrin-B2 reverse signals in arterial endothelial cells mediate propulsive effects that act coordinately with the repulsive effects of EphB4 in vein endothelial cells (37) (section 3.1). Consistent with this model, an artery to vein direction of sprouting angiogenesis has been observed in the avian yolk sac (43) (section 4.2).

2.4. Crosstalk With Other Angiogenic Pathways

Activation of EphA2 by ephrin-A1 has been shown to mediate the angiogenic effects of Tumor necrosis factor a (TNFa) both in vitro and in vivo (Fig. 3A). TNFa and other pro-inflammatory cytokines upregulate ephrin-A1 expression in endothelial cells, which in turn promotes activation of EphA2 (as shown by increased tyrosine phosphorylation of this receptor) and capillary morphogenesis (5,18,44,45). Furthermore, activation of EphA2 is required for corneal neovascularization induced by TNFa (5). TNFa regulates ephrin-A1 expression in endothelial cells through the p38 MAP kinase and JNK (46), and it will be interesting to examine whether these MAP kinases in turn regulate the transcription factor HoxB3 (section 2.1).

Consistent with a role in angiogenesis, ephrin-A1 is downregulated in human microvascular endothelial cells by treatment with the anti-angiogenic factor endostatin (47). Ephrin-A1 is also an important mediator of the angiogenic effects of VEGF, which instead upregulates ephrin-A1 expression (19). Studies using EphA2 Fc to block the interaction between endogenous ephrin-A1 and EphA2, or EphA2 antisense oligonucleotides to reduce EphA2 expression, have shown that the ensuing stimulation of EphA forward signaling plays a role in some of the angiogenic activities of VEGF, such as microvascular endothelial cell survival, migration and sprouting in vitro as well as the formation of new blood vessels in vivo in corneal neovascularization assays and Matrigel assays (14,15,19,48). In contrast, endothelial cell proliferation induced by VEGF, and the angiogenic effects of basic fibroblast growth factor (bFGF), seem to be independent of ephrin-A1 and EphA2 (5,19).

There is also crosstalk between endostatin and VEGF and the EphB/ephrin-B signaling pathways (Fig. 3B). Endostatin downregulates ephrin-B1 and ephrin-B2 as well as EphB4 in human dermal microvascular endothelial cells (47). In contrast, VEGF upregulates ephrin-B2 in cultured endothelial cells (49,50) and in vivo in arterial endothelial cells of the embryonic skin (49), in a subset of the blood vessels induced in corneal neovascularization assays (51), and in capillaries induced by VEGF transgenic expression in the mouse heart (52). A pathway responsible for ephrin-B2 expression likely involves Notch and TGFb signaling (53). Indeed, TGFb1 and activin-A can upregulate ephrin-B2 expression in mouse primary embryonic endothelial cells, similar to VEGF (49). Interestingly, loss-of-function studies in zebrafish embryos have shown that Notch signaling not only upregulates arterial markers like ephrin-B2

but also represses venous markers like EphB4 (54). In turn, EphB receptor activation by ephrin-B2 Fc has been shown to attenuate VEGF-induced HUVE cell proliferation and migration (26,37).

Other growth factors in addition to VEGF, as well as plating cells on a Matrigel substrate, have been reported to upregulate ephrin-B2 expression in endothelial cells (30,50). The other growth factors that have been shown to upregulate ephrin-B2 include VEGF-C, interleukin 6 and interleukin 8 in HUVE cells and hepatocyte growth factor and bFGF in human aortic and dermal microvascular endothelial cells. Activation of EphB4 by ephrin-B2 Fc in the aortic endothelial cells in turn inhibits the angiogenic effects of bFGF. This effect involves upregulation of syndecan 1 expression and shedding of the ectodomain of this proteoglycan from the cell surface (55). The overproduced soluble syndecan-1 ectodomain inhibits FGF receptor signaling, likely by sequestering bFGF away from its receptor. A further twist is that heparitinase, an enzyme that preferentially targets desulphated heparin, converts the soluble syndecan-1 ectodomain from an inhibitor to an activator of bFGF binding to its receptor. Interestingly, enzymes with activity similar to heparitinase are present in inflamed tissue, where they would be predicted to modify the effects of ephrin-B2 on FGF receptor signaling (section 5.3).

Phorbol myristate acetate (PMA) also promotes the assembly of renal microvascular endothelial cells into capillary-like tubes, and this effect involves activation of EphB1 and EphB2 by endogenously expressed ephrin-B1 (13). Ephrin-B1 levels are not changed by PMA treatment, however, suggesting another form of regulation that may involve ephrin-B1 clustering induced through phosphorylation by protein kinase C (PKC), a serine/threonine kinase that is activated by PMA.

The Tie2 receptor tyrosine kinase has also been shown to phosphorylate tyrosine residues in the cytoplasmic domain of ephrin-B1, at least in vitro, which may also modulate ephrin-B angiogenic activities (25). Ephrin-B2-EphB4 signaling in turn appears to increase the expression of Tie2 and its ligand, angiopoietin-1, because Tie2 and angiopoietin-1 are poorly expressed in ephrin-B2 knockout mice (39). Interestingly, the phenotype of the angiopoietin-1 and Tie2 knockout mice resembles that of ephrin-B2 and EphB4 knockout mice (chapter 16). This raises the intriguing possibility that ephrin-B2-EphB receptor signaling may mediate blood vessel remodeling at least in part by upregulating the expression of angiopoietin-1 and Tie-2 (section 4.2). In turn, however, angiopoietin-1 has been shown to downregulate ephrin-B2 expression in human dermal microvascular endothelial cells in culture (30) and EphB receptor activation by ephrin-B2 Fc counteracts angiopoietin-1-induced cell migration in HUVE cells (26). Furthermore, transgenic co-expression of angiopoietin-2 (another member of the angiopoietin family) and VEGF reduces the number of ephrin-B2-positive blood vessels, resulting in a fraction of blood vessels that express neither ephrin-B2 nor EphB4 (52).

Interestingly, pulmonary microvascular endothelial cells isolated from EphA2 knockout mice have normal ephrin-A1 expression but increased expression of EphB4 and ephrin-B2, suggesting compensatory mechanisms and crosstalk between Eph receptors and ephrins of the A and B subclasses (56). Although all these findings do not yet provide a cohesive picture, they nevertheless show that there are many forms of crosstalk within the Eph family and between the Eph system and other angiogenic signaling pathways, leading to complex networks of positive and negative feedback loops that coordinately regulate the properties of vascular cells.

3. Endothelial cell fate

Eph receptors and ephrins are expressed in stem cells and progenitor cells of different lineages, where they regulate the balance of proliferation and self-renewal versus differentiation, cell fate determination, and even cell death (1,57). Both EphB4 and ephrin-B2 are expressed in mouse undifferentiated embryonic stem cells and in early embryoid bodies derived by culturing those stem cells (58). Interestingly, EphB4 has been implicated in the differentiation of a common precursor cell, the hemangioblast, towards endothelial and hematopoietic cell lineages (58,59). When embryoid bodies are used to generate hemangioblast cells in vitro, mimicking developmental events occurring in the blood islands of the yolk sac, EphB4-deficient embryoid bodies display defects in hemangioblast development (58). EphB4 knockout embryoid bodies also produce reduced numbers of endothelial cells and have impaired ability to develop endothelial cells sprouts in the presence of angiogenic growth factors, suggesting a role for EphB4 in the assembly of the primitive vascular network. However, the deficiencies in the production of hemangioblast cells and in the assembly of capillary sprouts are corrected over time, suggesting that EphB4 facilitates these processes, but is not absolutely required. This may explain why major defects in the initial assembly of blood vessels by vasculogenesis have not been noted in the EphB4 knockout mice (section 4.2).

4. Angiogenic Remodeling of Embryonic Blood Vessels

4.1. Ephrin-A1 and EphA receptors

Ephrin-A1 is widely expressed in embryonic veins and arteries starting at very early stages of development (60,61), consistent with its angiogenic role in vitro and in angiogenesis assays in vivo (Fig. 2A; section 2.1). However, the role of ephrin-A1 in the formation of embryonic blood vessels remains to be determined. It was noted that ephrin-A1 expression is uneven in different blood vessels in the same organ and in different endothelial cells in the same blood vessel and

declines as development progresses (62), but the significance of these observations is unknown. Ephrin-A1 knockout mice will be an important tool to elucidate the function of this ephrin during development of the vascular system, once they become available. The EphA receptors that interact with ephrin-A1 in embryonic blood vessels have not been identified. Despite the expression of EphA2 at sites of adult angiogenesis and the critical role of this receptor in angiogenic responses, EphA2 has not been detected in the embryonic vasculature and EphA2 knockout mice do not exhibit overt defects in vascular development (20). Other EphA receptors present in the developing vasculature are EphA4 and EphA7, whose expression in the endothelium and mesenchyme of umbilical arteries is regulated by the Homeobox A13 (HoxA13) transcription factor (63). Deficient expression of EphA4 and EphA7 in HoxA13 knockout mice may contribute to the observed narrowing of the umbilical arteries and loss of stratification of vascular mesenchyme and endothelium in these vessels.

4.2. EphB4 and Ephrin-B2

Ephrin-B2 was the first molecular marker of arterial endothelial cells to be identified and EphB4 the first marker of venous endothelial cells (64,65) (Fig. 2B). These expression patterns of ephrin-B2 and EphB4, which are observed from the earliest stages of angiogenesis, revealed for the first time that the separate identity of venous and arterial endothelial cells is specified before blood flow is established. However, recent studies support the idea that the arterial or venous identity of endothelial cells remains plastic and can be regulated by local cues such as hemodynamic forces after onset of the embryonic circulation and laminar shear stress in cultured endothelial cells (43,66,67). Although ephrin-B2 and EphB4 have been increasingly used as markers of arterial versus venous vessel identity, recent data suggest that additional criteria should also be considered. For example, moderate hypoxia causes loss of arterial expression of ephrin-B2 and its possible upstream regulator, Delta-like 4, in the developing mouse retinal vasculature (68). However, other markers of arterial identity remain present suggesting that vessels can loose ephrin-B2 expression but still maintain arterial characteristics. There are also some exceptions to the arterial and venous segregation of ephrin-B2 and EphB4. For example, in human embryonic lung vasculature, EphB4 and ephrin-B2 are not segregated in veins and arteries as they are in the mouse (69).

Interestingly, VEGF secreted locally by peripheral nerves in the skin is responsible for the alignment of blood vessels with the nerves and for the arterial differentiation of these nerve-associated blood vessels, which involves upregulation of ephrin-B2 (49) (section 2.3). Less is known about the regulation of EphB4 expression in the vasculature, although the HoxA9 transcription factor has been reported to upregulate EphB4 in endothelial cells, at least in vitro (41) (section 2.3). EphB4 may also positively regulate its own expression because homozygous

knockout mice in which the b-galactosidase reporter gene is expressed in place of EphB4 show lower levels of b-galactosidase compared to heterozygous mice, which have one functional EphB4 allele (65).

The phenotype of ephrin-B2 and EphB4 knockout mice does not reveal prominent defects in the initial assembly of blood vessels during vasculogenesis (39,64,65). The networks of evenly sized capillaries of the primary vascular plexus in the yolk sac and in the head of these mice appear to form normally, although it is not known whether subtle delays may occur in the generation of endothelial cells (section 3). Ephrin-B2 and EphB4 are, however, required for normal development of the dorsal aorta and cardinal veins, which are assembled from coalescing endothelial precursor cells by vasculogenesis (25,64,65).

The most notable early angiogenic defect in the ephrin-B2 and EphB4 knockout mice is a failure to remodel the primary vascular plexus into a mature, functional vasculature consisting of large and small interconnected branches (64,65). For example, the vasculature in the yolk sac and in the head persists as an immature network of evenly sized capillaries in the absence of ephrin-B2 or EphB4, and fails to undergo reorganization. This reorganization normally involves sprouting of new blood vessels and pruning of existing vessels as well as fusion and splitting of vessels and, as recently shown by time-lapse imaging, disconnection of small arterial vessels and their reconnection to form new junctions with the venous network (43,70). EphB-ephrin-B bidirectional signaling could in principle regulate all of these processes (section 2.3). Remarkably, despite the endothelial expression of ephrin-B2 only in arteries and EphB4 mainly in veins, the vascular defects in the ephrin-B2 and EphB4 knockout mice are quite similar and affect both arteries and veins. In addition, decreased capillary formation in mice overexpressing ephrin-B2 in endothelial cells suggests that a precise level of ephrin-B2 expression is essential for arterial-venous capillary boundary formation (71). Interestingly, exposure of the developing mouse retinal vasculature to decreased (10%) oxygen levels causes loss of ephrin-B2 and inappropriate separation of the arterial and venous networks, supporting a role for ephrin-B2-EphB4-mediated cell repulsion in the normal segregation of arteries and veins (68). Ephrin-B2 expressed in the intersomitic arteries and EphB4 expressed in the intersomitic veins are also required for the subsequent angiogenic remodeling of the intersomitic vasculature (65,72).

The similar phenotypes of the ephrin-B2 and EphB4 knockout mice led to the proposal that bidirectional signals mediated by these molecules between arterial and venous endothelial cells are critical for angiogenic remodeling (64,65). For example, repulsive interactions could prevent arteries and veins from fusing during angiogenic remodeling, while allowing the fusion of vessels of the same type. Repulsive signals could also be important in establishing the proper balance of arteries and veins in capillary beds as well as in the formation and maintenance of the arteriovenous boundary. Although only limited interfaces between the arterial and venous sides

exist in the mature vasculature, there is some evidence that extensive transient connections form during angiogenic vascular remodeling in the embryo (72). Thus, proper signaling at the arterial-venous boundaries may be essential for remodeling of the entire network to occur.

In vitro studies suggest that unidirectional EphB4 forward signaling is sufficient to segregate EphB4-expressing endothelial cells from ephrin-B2-expressing cells by restricting cell intermingling (37) and mouse knock-in studies have shown that ephrin-B2 reverse signaling is not required for angiogenic vascular remodeling in the early embryo. Replacement of wild-type ephrin-B2 with an ephrin-B2 mutant lacking all tyrosine phosphorylation sites in the cytoplasmic domain or with a mutant lacking the carboxy-terminal PDZ domain binding site rescues the early vascular defects observed in ephrin-B2 knockout mice (73). Furthermore, a form of ephrin-B2 in which the entire cytoplasmic domain is replaced by b-galactosidase supports normal angiogenesis in the early embryo, similar to wild-type ephrin-B2 (74). These data indicate that the function of ephrin-B2 in the early arteries is to stimulate EphB forward signaling, whereas reverse signaling appears to be dispensable. Different results obtained with a mutant ephrin-B2 in which most of the cytoplasmic domain was replaced by the short HA tag may be explained by a partially defective ability of this mutant to elicit EphB reverse signaling (39,73). These data indicate that the main function of ephrin-B2 in early embryonic angiogenesis is as a ligand that stimulates EphB receptor forward signaling, while its intrinsic reverse signaling function is dispensable. Although in the embryo ephrin-B2 is expressed not only in endothelial cells but also in adjacent mesenchymal cells, the phenotype of conditional knockout mice that lack ephrin-B2 only in endothelial and endocardial cells (due to Cre expression driven by the Tie2 promoter) is indistinguishable from the phenotype of mice lacking ephrin-B2 in all cells (72). Taken together, these data indicate that the essential function of ephrin-B2 in arterial endothelial cells is to stimulate EphB forward signaling, which is sufficient to mediate remodeling of both arteries and veins.

Although repulsive effects mediated by unidirectional EphB4 signaling in veins at the boundaries with arteries likely play a critical role in blood vessel maturation in the embryo, additional mechanisms may contribute to the observed arterial defects of the mutant mice. For example, ephrin-B2 could affect arterial endothelial cells by stimulating forward signaling by the EphB4 receptor present at low levels in scattered arterial endothelial cells (65). Arterial defects in the ephrin-B2 knockout mice may also be a secondary consequence of defective blood flow due to an abnormal heart and/or abnormal veins (65). Although defects in vascular remodeling begin in the ephrin-B2 and EphB4 knockout mice before the blood circulation is established and continue to be present at later stages in a subset of embryos that maintain blood flow (64,65), perfusion could nevertheless be perturbed in these embryos. Interestingly, recent experiments have shown that hemodynamic forces and laminar shear stress can modify the expression of

arterial markers such as ephrin-B2 (43,67). Another intriguing possibility is that ephrin-B2 stimulates EphB4 forward signaling pathways that regulate expression of other molecules that are important for angiogenic remodeling of the embryonic vasculature, such as angiopoietin-1 and its receptor Tie2 (39) (section 2.4) (chapter 16). However, given the effects of ephrin-B2 and EphB4 on endothelial cell shape, attachment, migration and proliferation in vitro (section 2.3), it seems probable that these molecules must exert some direct effects on the behavior of endothelial cells in vivo independently of the angiopoietin-1/Tie2 system.

Whether ephrin-B2 also plays an essential role in vascular support cells, in addition to endothelial cells, awaits examination of conditional knockout mice lacking ephrin-B2 only in these cells. A clue as to the function of ephrin-B2 in vascular smooth muscle cells may be the finding that ephrin-B2-expressing stromal cells promote growth and angiogenic sprouting of ephrin-B2-expressing endothelial cells in a co-culture explant system (75). A functional role for non-endothelial ephrin-B2 in the ascending aorta is also suggested by the abnormalities in the recruitment of smooth muscle cells that have been observed in mice ubiquitously overexpressing ephrin-B2 but not in mice overexpressing ephrin-B2 only in endothelial cells (71).

4.3. Other EphB Receptors and Ephrin-Bs

In situ hybridization studies have revealed the presence of additional EphB and ephrin-B molecules in the developing vasculature (25). Ephrin-B1 is expressed in both arterial and venous endothelial cells. However, it cannot compensate for the loss of ephrin-B2, perhaps due to its poor ability to activate EphB4. The EphB3 receptor is expressed in veins and in the aortic arches, while EphB2 is not expressed in endothelial cells but it is present in surrounding mesenchymal cells. These expression patterns indicate that ephrin-B-EphB interactions are not restricted to the arterial-venous boundaries but can also occur between endothelial cells in the same vessel and at endothelial-mesenchymal interfaces and involve more than just ephrin-B2 and EphB4 (Fig. 2B). For example, endothelial ephrin-B2 could also function by stimulating EphB2 expressed in vascular support cells surrounding the endothelial cells.

Genetic evidence indicates that these additional interactions are functionally important. Although mice lacking either EphB2 or EphB3 do not exhibit overt vascular defects, some of the double knockout mice lacking both receptors have vascular defects similar to those of the ephrin-B2 (and EphB4) knockout mice (25). Thus, EphB2 and EphB3 also play a role in the maturation of the primitive vascular plexus and the formation of major vessel primordia, presumably through interactions with ephrin-B2 (section 4.2) and possibly ephrin-B1. Defects in the association of arterial endothelium with support cells in the yolk sac of ephrin-B2 knockout embryos suggest a role for ephrin-B2 in the interaction of endothelial cells with surrounding cells (64). A role for endothelial ephrin-B2 in mediating interactions with EphB2-positive surrounding

cells in the neural tube has been suggested to promote the angiogenic sprouting of the blood vessels that penetrate into the neural tube, based on the finding that the neural tube remains avascular in ephrin-B2 knockout mice (64). However, in EphB2/EphB3 double knockout mice the neural tube is vascularized, suggesting either that another receptor for endothelial ephrin-B2, such as EphB1 or EphA4, is the counterpart for ephrin-B2 or that a different mechanism requiring endothelial ephrin-B2 mediates sprouting angiogenesis in the neural tube (25).

EphB2/EphB3 double knockout mice, and ephrin-B2 knockout mice in at least some genetic backgrounds, also exhibit aberrant sprouting of intersomitic vessels into the adjacent somitic tissue (25). This evidence suggests that forward signaling by EphB3 and EphB4, which are expressed in the mouse intersomitic veins, mediates repulsive signals in response to ephrin-B2 expressed in the posterior portion of the somites and the intersomitic arteries, two structures that flank an ephrin-B2-free path where the intersomitic veins extend (25,71). Consistent with this, widespread expression of ephrin-B2 in the mouse embryo under the control of a ubiquitous promoter, which abolishes discontinuous presentation of endogenous ephrin-B2, causes abnormalities in the projection of intersomitic veins (71). A similar phenotype is caused in Xenopus embryos by ectopic expression of ephrin-B ligands, or overexpression of a dominant negative form of EphB4 that impairs the ability of endogenous endothelial EphB4 to signal (76). Taken together, these data support a model where EphB receptor forward signaling in intersomitic venous endothelial cells inhibits the formation of vascular sprouts extending into ephrin-B territories.

In summary, it appears that a balance of several EphB receptors and ephrin-B ligands expressed in endothelial and vascular support cells is required to achieve proper blood vessel sprouting and remodeling during embryonic development.

4. Lymphatic Vessels

Recent findings have shown that ephrin-B2 and EphB4 are also expressed in lymphatic blood vessels, where they play a critical role in the formation of a functional vascular tree (73). Analysis of LacZ reporter mice revealed that ephrin-B2 is expressed in the endothelial cells of collecting lymphatic vessels, which have smooth muscle cell coverage and contain valves, and EphB4 is widely expressed throughout the lymphatic networks, including capillaries. Interestingly, ephrin-B2 reverse signaling is important in many of the lymphatic vessels. Knock-in mice engineered to express a mutated ephrin-B2 lacking the PDZ domain-binding site have accumulation of chylous lymphatic fluid in body cavities and exhibit major lymphatic defects. For example, the primary lymphatic plexus in the skin (which expresses both ephrin-B2 and EphB4) forms normally but there are defects in subsequent sprouting of new capillaries

(expressing only EphB4) and in vascular remodeling. In addition, collecting lymphatic vessels are hyperplastic and lack the luminal valves that allow proper lymphatic drainage. In contrast, knock-in of a mutated form of ephrin-B2 lacking all the cytoplasmic tyrosine phosphorylation sites almost fully compensates for the lack of wild type ephrin-B2. These data indicate that ephrin-B2 interaction with PDZ domain-containing proteins is required for normal development of the lymphatic vasculature, whereas ephrin-B2 tyrosine phosphorylation and interaction with SH2 domain-containing proteins are dispensable. Consistent with this idea, several known ephrin-B2 binding proteins that contain PDZ domains were found to have altered subcellular distribution in lymphatic vessels expressing the mutant ephrin-B2 without the PDZ domain-binding site. Ephrin-B2 reverse signaling therefore seems to play a more important role in lymphatic vascular morphogenesis than in blood vessel morphogenesis.

5. Adult Vasculature

Given the importance of Eph receptors and ephrins in the formation of the embryonic vasculature, it is not surprising that these molecules have also been implicated in physiological and pathological forms of postnatal angiogenesis.

5.1. Quiescent vasculature

Ephrin-A1 is downregulated during embryonic development and is not detectable in adult quiescent vasculature (60), but some EphA receptors are expressed in normal adult blood vessels. For example, EphA7 has been detected in the blood vessels of the liver septa and in blood vessels of the renal parenchyma (77). However, the function of these receptors in the adult vasculature and the identity of their ephrin-A ligand counterpart are not known.

A stable molecular difference between arteries and veins persists in the adult quiescent vasculature, where ephrin-B2 remains expressed in arterial endothelial cells (51,78). EphB4 remains expressed in small diameter venous microvessels and capillaries as well as in large veins such as the vena cava, where this receptor exhibits a patchy heterogeneous expression. In addition, however, low levels of ephrin-B2 have been detected in some cells of adult veins, such as the vena cava, and EphB4 has been detected in some arteries. Another receptor for ephrin-B2 in the adult vasculature is EphB1, which in adult kidney glomeruli is expressed at higher levels than during development (17). Thus, interactions between ephrin-B2 and EphB receptors likely continue to play a role in the maintenance of a mature vessel configuration.

An increasingly important role of ephrin-B2 at later stages of vascular development may be to regulate vascular smooth muscle cells. The endothelial expression domain of ephrin-B2 expands as development progresses to include the vascular smooth muscle cells of many arteries

where this ligand continues to be expressed in the adult (51,78). Interestingly, ephrin-B2 is upregulated in vascular smooth muscle cells only after they have already lined blood vessels and expression starts from the smooth muscle cells directly in contact with endothelial cells and gradually progresses towards more external regions (51).

5.2. Physiological angiogenesis

EphA2 is one of the key Eph receptors that play a role in postnatal angiogenesis, even though it does not seem to be involved in angiogenesis during embryonic development (section 4.1). With regard to the B class, ephrin-B2 continues to be expressed in the arterial vasculature at sites of secondary angiogenesis in the embryo, such as the heart, neural tube, kidney and lung. Ephrin-B2 expression also persists at sites of adult angiogenesis, such as the ovarian follicles and the corpus luteum in the female reproductive system (78). Here, ephrin-B2 presumably plays an important role in the continuous vascular remodeling that occurs during the estrous cycle. It has also been recently proposed that the ephrin-B/EphB system plays a role in connecting the blood vessels of the human placenta to the maternal circulation, a process mediated by fetal cytotrophoblast cells that invade the uterine wall to reach arterioles and remodel them (79). During their differentiation, the cytotrophoblast cells lose EphB4 expression and acquire ephrin-B1 and ephrin-B2 expression, which results in repulsive signals and reduced responsiveness to attractive cytokines in response to EphB4. These repulsive signals likely limit interaction of the cytotrophoblast cells with uterine veins, which express EphB4, and promote selective invasion and remodeling of the uterine arterioles, which express ephrin-B2.

5.3. Inflammation and wound healing

Ephrin-A1 and EphA2 are involved in angiogenesis in response to inflammatory cytokines such as tumor necrosis factor a (5) (section 2.4). Ephrin-B2 and EphB4 may also play a role in adult inflammatory neovascularization because according to recent data they are upregulated in pyogenic granuloma of human gingiva, which is a benign inflammatory lesion (80). Furthermore, ephrin-B2 expression is upregulated in HUVE cells not only by VEGF but also by the inflammatory cytokines interleukin-6 and interleukin-8 (50). Ephrin-B2 also becomes expressed in a subset of blood vessels in the skin during wound healing (51) and, presumably, plays a general role in restoring blood vessels at sites of tissue injury. Interestingly, one of the effects of EphB4 activation by ephrin-B2 in a murine would healing model is upregulation of syndecan-1. This likely promotes angiogenesis in vivo if it is accompanied by increased secretion of pro-inflammatory enzymes such heparanases, which could switch syndecan 1 from an inhibitor to a stimulator of bFGF binding to its receptor (55) (section 2.4).

Hypoxia has recently been shown to upregulate expression of ephrin-B2 and its receptor EphB4, as well as ephrin-A1 and EphA2, in the blood vessels of the mouse skin (81) (Fig. 3). Ephrin-B2 is also upregulated in the new arterial vessels that grow to restore circulation after tissue ischemia in the limb (30). Furthermore, hypoxia upregulates ephrin-B2 in human umbilical arterial endothelial cells in vitro (82). Therefore, hypoxia may link Eph receptor and ephrin expression to adult neovascularization under both physiological and pathological conditions.

5.4. Tumor angiogenesis

The ephrins and Eph receptors that have been most prominently detected in tumor blood vessels are ephrin-A1, ephrin-B2 and EphA2 (Fig. 4). An emerging theme is that the interplay between Eph receptors and ephrins expressed in endothelial and tumor cells plays an important role in tumor angiogenesis (38,48). Interactions between tumor cells and endothelial cells may occur particularly during the sprouting and remodeling of new blood vessels. In addition, however, the endothelial cells in tumor blood vessels can be surrounded by a discontinuous basement membrane or lack supporting smooth muscle cells (83). Hence, tumor endothelial cells have the opportunity to interact with the tumor cells (Fig. 4). In addition, tumor cells are sometimes interspersed among endothelial cells and participate in lining the blood vessel wall and can even form blood vessel-like channels (83,84). Interestingly, hypoxia has been shown to upregulate ephrin-A1, EphA2, ephrin-B2 and EphB4 in both endothelial and tumor cells (81). In at least some tumor cells, this effect is mediated by the transcription factor Hypoxia-inducible factor-1a (HIF-1a), which is also known to upregulate VEGF expression. It is therefore possible that VEGF secreted by the tumor cells in turn stimulates the endothelial expression of ephrins in the tumor vasculature, thus resulting in the coordinated upregulation of Eph receptor and ephrin expression in both tumor cells and the tumor vasculature in hypoxic regions.

5.4.1. Ephrin-A1 and EphA2

Both ephrin-A1 and EphA2 are upregulated in the vasculature of different types of tumors, while neither protein has been detected in quiescent adult vasculature (14,21) (Fig. 4A). The factors upregulating EphA2 expression in the tumor vasculature have not been identified. On the other hand, there is evidence that inflammatory cytokines and hypoxia contribute to upregulate ephrin-A1 expression in the blood vessels of a tumor and the surrounding tissue leading to EphA2 activation (21,48,81) (section 2.1). Furthermore, VEGF produced by tumor cells likely plays an important role in the upregulation of ephrin-A1 in tumor endothelial cells (19). Indeed, conditioned medium from islet carcinoma cells, which are known to produce VEGF, promotes EphA2 tyrosine phosphorylation and the migration of HUVE endothelial cells in transwell migration assays (48). This pro-migratory effect is blocked by VEGF-neutralizing antibodies and

also by EphA2 Fc, a soluble EphA2 antagonist. These data implicate ephrin-A1 upregulation and the consequent EphA2 activation in mediating some of the effects of VEGF on tumor angiogenesis (Fig. 3A).

In many tumors ephrin-A1 and EphA2 are also expressed in the tumor cells (Fig. 4A), and therefore a complex interplay between EphA2 and ephrin-A1 expressed in tumor cells and tumor blood vessels likely takes place, with positive effects on angiogenesis. Supporting this idea, EphA2 overexpression in cancer cells has been recently reported to correlate with high microvessel counts in human colorectal cancer specimens (85). In addition, mammary and pancreatic tumor cell lines that express ephrin-A1 attract endothelial cells in co-culture transwell migration assays (14,48). A gain-of-function EphA2 mutant enhances the tumor cell-induced endothelial cell migration, while a dominant negative mutant and soluble EphA2 Fc inhibit migration (14,48). Furthermore, microvascular pulmonary endothelial cells from EphA2 knockout mice migrate less efficiently in response to 4T1 mammary tumor cells and endothelial cells from wild type mice migrate less efficiently in response to tumor cells with decreased ephrin-A1 expression (56). These data suggest that VEGF and ephrin-A1/EphA2 cooperate in promoting blood vessel recruitment by the tumor. A possible scenario is that VEGF provides a long-range signal that upregulates ephrin-A1 in endothelial cells leading to activation of endothelial EphA2, whereas ephrin-A1 on tumor cells provides a contact-dependent signal by interacting with endothelial EphA2 (14).

Soluble EphA2 antagonists – such as EphA2 Fc and EphA3 Fc – have been shown to inhibit tumor angiogenesis and progression in mouse xenograft models of breast and pancreatic cancer when administered either systemically or subcutaneously in the vicinity of the tumor (14,15,19,48). Among the effects documented are a decrease in cell proliferation and an increase in apoptosis of both the tumor cells and the endothelial cells (14,15). The effects on the tumor cells, however, may be secondary to decreased vascularization because EphA2 Fc has no direct effect on the survival of mammary and pancreatic tumor cells in culture (14,15). Consistent with effects on blood vessels, EphA2 Fc inhibits mammary and pancreatic tumor-induced angiogenesis in an in vivo cutaneous window angiogenesis assay, in which a small tumor placed in a subcutaneous chamber becomes vascularized by host blood vessels (14). EphA Fc fusion proteins also inhibit the formation of premalignant lesions and reduce tumor volume in the RIP-Tag transgenic mouse pancreatic cancer model (48). Furthermore, tumor growth is impaired and vascular density is decreased when mouse 4T1 mammary adenocarcinoma cells are implanted in EphA2 knockout mice, demonstrating the importance of EphA2 in tumor blood vessels and the tumor microenvironment (56). Taken together, the available evidence suggests that EphA2 forward signaling in tumor blood vessels is critical for tumor growth and that inhibition of

endothelial EphA2 forward signaling is the main mechanism underlying the anti-cancer effects of EphA receptor Fc fusion proteins.

5.4.2. Ephrin-B2 and EphB4

Ephrin-B2 has been detected in the vasculature of a variety of tumor types, where it is expressed in a proportion of the endothelial cells (38,51,78,86) (Fig. 4B). This suggests that tumor capillaries can have arterial or venous identity, contrary to previous beliefs based on morphological criteria. EphB4 expression in tumor endothelial cells remains to be characterized, however, and it is not known whether the ephrin-B2-negative tumor blood vessels express EphB4 and whether a complementary expression of endothelial ephrin-B2 and EphB4 is important for the formation of new vascular networks in tumors. Ephrin-B2 expression in tumor blood vessels is likely upregulated by VEGF (section 2.4) and hypoxia (81,82). However, expression of ephrin-B2 was not detected in the smooth muscle cells associated with the vasculature of Lewis lung carcinomas grown subcutaneously in mice (78).

Enhanced expression of the EphB4 receptor ectodomain on the tumor cell surface has been shown to promote tumor growth by promoting ephrin-B2 reverse signaling in the tumor blood vessels (38). Interestingly, increased EphB4 ectodomain expression in the tumor cells increases the size of the blood vessels and the blood content of the tumors, consistent with the enlarging effects of EphB4 Fc on the chicken allantoic arteries and the enlarged ear skin blood vessels in transgenic mice overexpressing ephrin-B2 in endothelial cells (43,71). Conversely, A375 melanoma cells secreting soluble monomeric EphB4 ectodomain form much smaller tumors when injected subcutaneously in nude mice, presumably because the EphB4 ectodomain blocks the interaction between EphB2 on the surface of the tumor cells and endothelial ephrin-B2 (87). Indeed, tumor regions where the EphB4 ectodomain was present at highest levels had low microvessel densities and more collapsed vessels lacking a lumen.

Although ephrin-B2 reverse signaling does not appear to be required during early embryonic angiogenesis, angiogenic effects of ephrin-B2 in tumors are consistent with the pro-angiogenic effects of ephrin-B2 reverse signaling in cultured endothelial cells (36-38). Ephrin-B2 reverse signaling can also regulate the vasculature in vivo. For example, defects in the postnatal lung vasculature have been reported in mice expressing mutated ephrin-B2 lacking the PDZ domain-binding site (73) and EphB4 Fc treatment of the allantoic membrane blood vessels of the E4 chicken embryo, which have already undergone remodeling into a network of large and small vessels, causes retraction or regression of venules, enlargement of arteries and formation of arterial-venous shunts (43). Furthermore, defects in capillary architecture in the kidney glomeruli and the mammary gland of mice overexpressing EphB4 in epithelial cells support a role for ephrin-B2 reverse signaling in endothelial cells stimulated by EphB4 expression in surrounding

cells (88). Interestingly, ephrin-B2 is upregulated in Kaposi's sarcoma, an angioproliferative tumor derived from endothelial cells, and is required for the viability of the tumor cells (50).

Additional evidence suggests that endothelial EphB receptors also play an important role in tumor angiogenesis. For example, ephrin-B1 is upregulated in hepatocellular carcinomas and promotes hepatocellular tumor growth in a mouse xenograft model without affecting the growth of the tumor cells in culture (27). Thus, ephrin-B1 on the surface of the tumor cells may be involved in tumor progression in vivo by promoting tumor angiogenesis through endothelial EphB receptors (Fig. 4B). Consistent with this hypothesis, the number of blood vessels in tumor xenografts is increased by ephrin-B1 expression in the tumor cells and ephrin-B1 Fc enhances HUVE cell migration and proliferation in vitro (27). Interestingly, treatment of the E4 chicken embryo allantois with ephrin-B2 Fc causes morphological effects in the vasculature (43). These morphological changes, presumably mediated by EphB4, include increased branching of veins, dramatic enlargement of both veins and arteries and formation of arterious-venous shunts. However, despite increasing blood vessel density, ephrin-B2 overexpression in human colorectal cancer cells decreases tumor growth and blood perfusion in a mouse xenograft model (89). Thus, additional studies are required in order to fully elucidate the molecular mechanisms by which Eph receptors and ephrins affect tumor progression by contributing to tumor vascularization.

6. Targeting Eph Receptor-Ephrin Interactions to Modulate Angiogenesis

In conditions where angiogenesis is part of the disease pathology, it is therapeutically desirable to inhibit it. A number of receptor tyrosine kinases expressed in endothelial cells are validated targets for anti-angiogenic therapies (Section III of Volume 2). Eph receptors and ephrins are also emerging as new attractive targets. EphA receptor Fc fusion protein and soluble monomeric forms of EphB4 have been successfully used as antagonists in animal tumor models (14,19,48,87). Furthermore, intravitreal injection of ephrin-B2 Fc or EphB4 Fc, which presumably perturb the function of the corresponding endogenously expressed proteins, reduce the pathological neovascularization occurring in a mouse model of oxygen-induced retinopathy (90). Thus, ephrin-B2 and EphB4 might be useful targets for therapies to treat retinopathy of prematurity and the abnormal retinal vascularization characterizing macular degeneration and diabetic retinopathy. Although Fc fusion proteins are quite stable when administered systemically in vivo, Eph receptor- and ephrin-based agents lack selectivity because of the promiscuity of Eph receptor-ephrin binding (section 1.3). More selective Eph receptor-targeting reagents that have been developed include antagonistic peptides that target the ephrin-binding site of individual Eph receptors (91,92), siRNAs and anti-sense oligodeoxynucleotides (93-95).

Peptides as well as antibodies that target extracellular regions of Eph receptors and ephrins could also be used to deliver anti-angiogenic drugs.

In other conditions that are characterized by ischemia, such as heart disease, stroke and wound healing, it may be desirable to use reagents that enhance the pro-angiogenic activities of Eph receptors and ephrins, such as activating peptides or antibodies (96,97). Ephrins or Eph receptor ectodomains incorporated into fibrin matrices could also be useful to promote angiogenesis, as shown for ephrin-B2 (98). Targeting Eph receptors and ephrins to stimulate or inhibit angiogenesis is an area just beginning to be explored and where rapid developments are expected.

7. Perspectives

The last few years have seen the discovery of Eph receptors and ephrins as new families of angiogenic factors that can discriminate between arteries and veins and that play a role not only in endothelial cells but also in the surrounding support cells. EphB4 and ephrin-B2 are expressed in angiogenic vasculature during embryonic development, at sites of adult neovascularization, and also in the mature quiescent vasculature. These expression patterns suggest diverse roles in neovascularization and the maintenance of mature blood vessels. The EphA2 receptor is also expressed at sites of adult neovascularization but, surprisingly, it has not been detected in normal developing vasculature or quiescent vasculature. This expression pattern makes EphA2 a particularly attractive target for selective anti-angiogenic therapies. On the other hand, ephrin-A1 is in developing but not adult vasculature. Therefore, additional A class Eph receptors and ephrins with angiogenic activities likely remain to be discovered. Antibodies with well-defined specificities will be critical in order to accurately map the expression patterns of different Eph receptors and ephrins in developing and postnatal vasculature. Despite the remarkable progress made so far, much remains to be learned about the mechanisms and signaling pathways used by Eph receptors and ephrins to regulate blood and lymphatic vessels. Areas of particular interest for the future will be to better characterize the roles of Eph receptors and ephrins in the expansion versus the differentiation of vascular stem cells and the role of different levels of hypoxia in regulating the expression of Eph and ephrin genes in endothelial cells and the surrounding tissue. It will also be important to better understand the complex interplay between Eph receptors and other families of angiogenic factors. New therapeutic approaches for targeting Eph receptors and ephrins to promote or inhibit angiogenesis are undoubtedly also forthcoming.

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Figure Legends

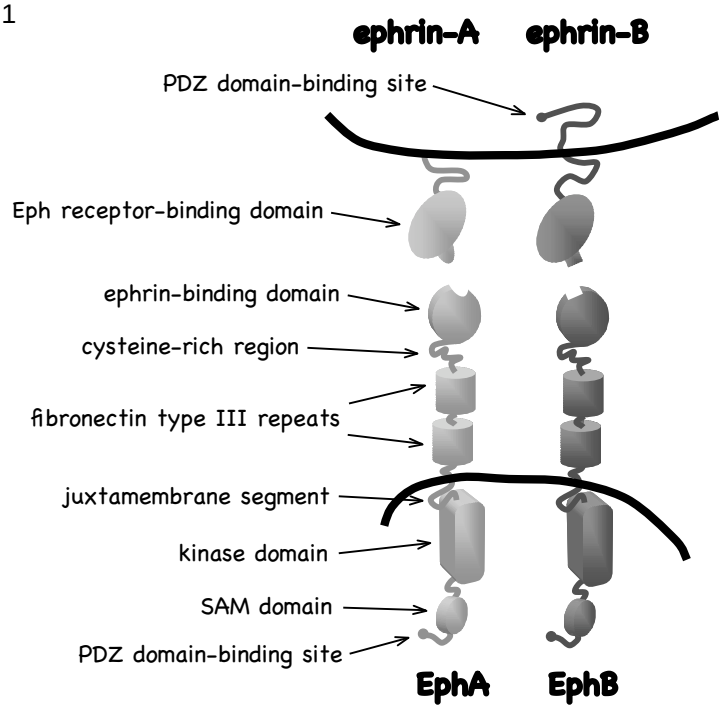
Figure 1. Prototypical domain structure of Eph receptors and ephrins. The different domains are indicated and the plasma membrane is represented by a thick black line.

Figure 2. Eph receptors and ephrins mediate interactions between vascular cells. (A) Ephrin-A1 is expressed in embryonic endothelial cells, in postnatal angiogenic endothelial cells, and in endothelial cells in culture. In postnatal and cultured endothelial cells, ephrin-A1 mediates angiogenic effects by activating the EphA2 receptor, which is co-expressed in the endothelial cells. The EphA receptor that is activated by ephrin-A1 in embryonic endothelial cells has not been identified. In culture, endothelial ephrin-A1 also affects the properties of vascular smooth muscle cells, which express the EphA4 receptor, suggesting that a similar interaction may affect vascular smooth muscle cells in vivo. **(B)** Ephrin-B2 is expressed in arterial endothelial cells (light gray) and EphB4 is expressed in venous endothelial cells (dark gray). Thus, this B class receptor and ephrin can mediate interactions between arterial and venous vasculature. Ephrin-B1 and other EphB receptors are also expressed in embryonic endothelial cells and in cultured endothelial cells, and could mediate interactions between adjacent endothelial cells of the same type. EphB2 and ephrin-B2 are expressed in vascular smooth muscle cells and pericytes and thus could mediate interactions between these vascular support cells and endothelial cells.

Figure 3. Various factors that regulate Eph receptor and ephrin expression in endothelial cells. Thin black arrows and bars indicate upregulation or downregulation of expression levels, respectively. Thick gray arrows indicate increased angiogenic responses. Activated (tyrosine phosphorylated) EphB receptors mediate angiogenic responses through either attractive or repulsive effects depending on the conditions, the endothelial cell type, and the receptor involved (see text for details). P, tyrosine phosphorylation.

Figure 4. Eph receptors and ephrins mediate interactions between tumor cells and endothelial cells. (A) Tumor endothelial cells as well as many tumor cells express both ephrin-A1 and EphA2. EphA2 signaling in tumor endothelial cells, which could be elicited by ephrin-A1 expressed in either the endothelial cells or the tumor cells, has been shown to promote tumor angiogenesis. **(B)** Ephrin-B2 is expressed in tumor endothelial cells and mediates pro-angiogenic reverse signals when interacting with EphB receptors expressed in tumor cells. Ephrin-B1 on tumor cells has also been shown to promote tumor angiogenesis, presumably by interacting with an EphB receptor expressed in tumor vasculature, which remains to be identified.

Figure 1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ephrin-B
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Eph receptor-binding domain
ephrin-binding domain
cysteine-rich region
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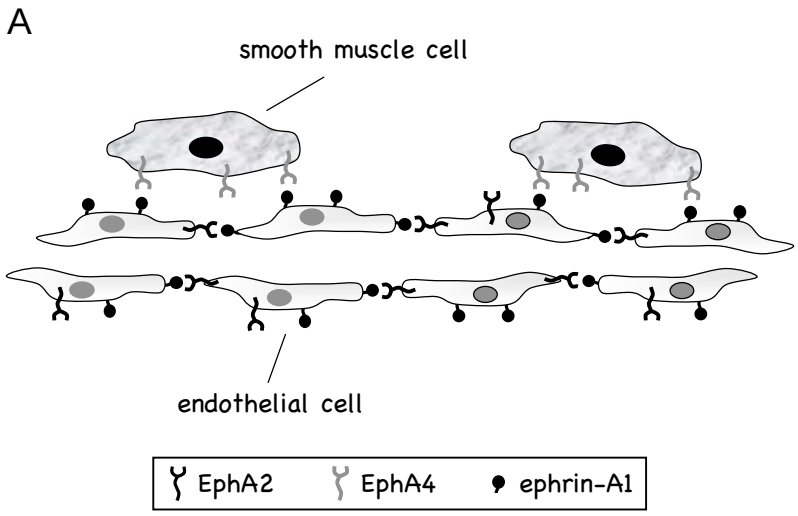
kinase domain
SAM domain
PDZ domain-binding site
EphA
EphB

Figure 2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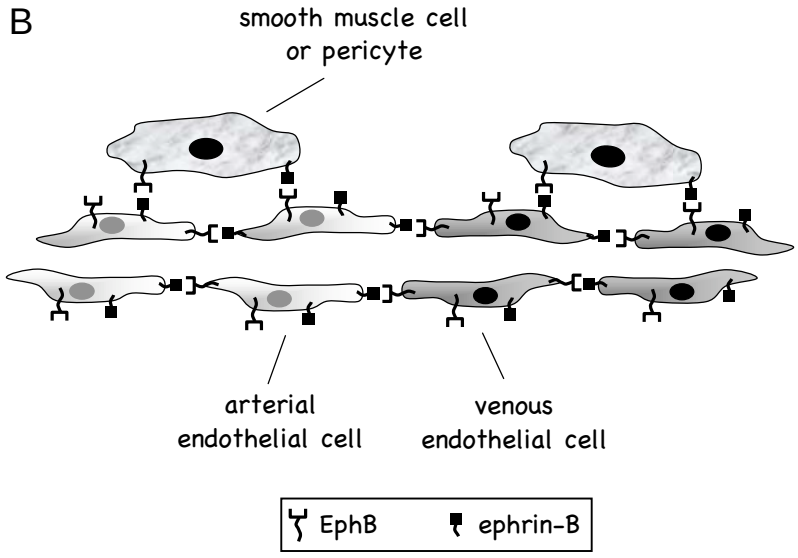
endothelial cell
EphA2
EphA4
ephrin-A1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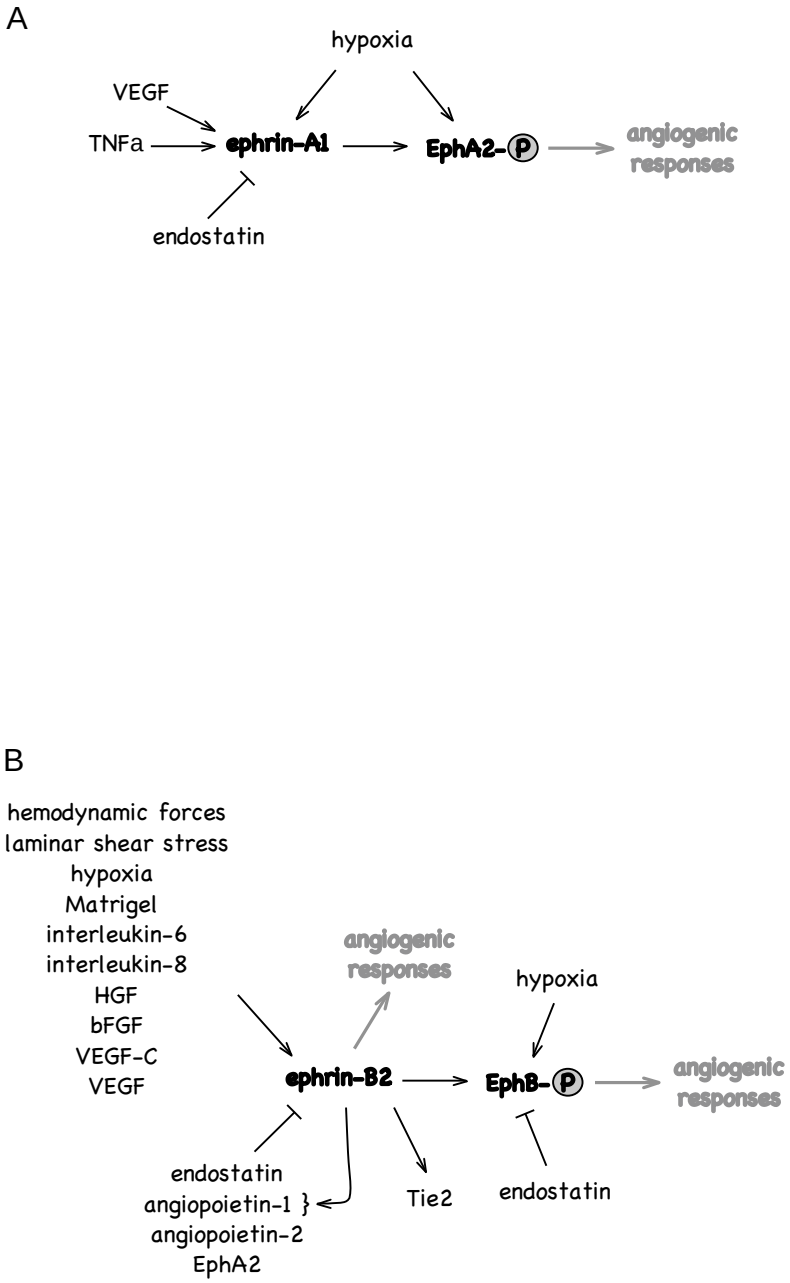
smooth muscle cell
or pericyte
arterial
endothelial cell
venous
endothelial cell
EphB
ephrin-B

Figure 3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TNFa
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EphA2-P
angiogenic responses
endostatin
B
hemodynamic forces
laminar shear stress
hypoxia
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interleukin-6
interleukin-8
HGF
bFGF
VEGF-C
VEGF
angiogenic responses
hypoxia
ephrin-B2
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angiogenic responses
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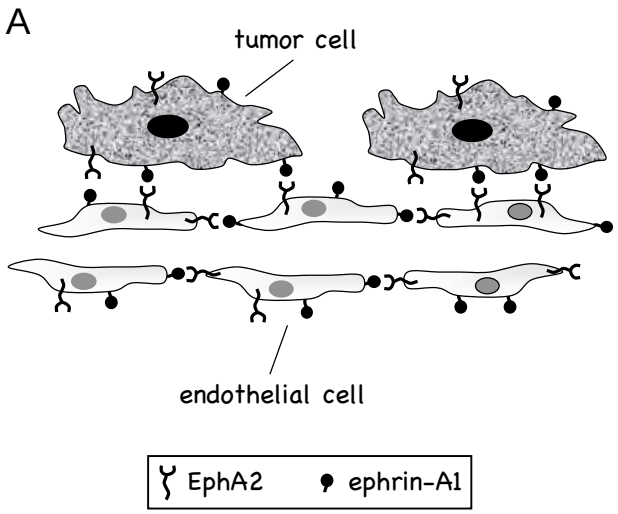
angiopoietin-1 }
angiopoietin-2
EphA2
Tie2
endostatin

Figure 4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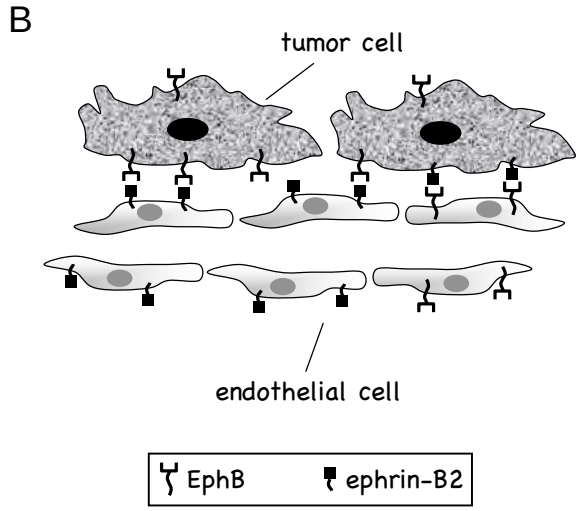
endothelial cell
EphA2
ephrin-A1


B
tumor cell
endothelial cell
EphB
ephrin-B2